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(54) Title: ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF

## (57) Abstract

The present invention provides a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein and a nucleotide sequence encoding an activity inducing domain of a second protein. Also provided is a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native anthrax PA protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target. Proteins encoded by the nucleic acid of the invention are also provided, as well as a method for delivering an activity to a cell using such fusion proteins. The invention also provides proteins including an anthrax protective antigen which has been mutated to replace the trypsin cleavage site with residues recognized specifically by the HIV-1 protease.

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## ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF

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This application is in a continuation in part application of Serial No. 08/021,601 filed February 12, 1993.

## BACKGROUND OF THE INVENTION

10       The targeting of cytotoxic or other moieties to specific cell types has been proposed as a method of treating diseases such as cancer. Various toxins including *Diphtheria* toxin and *Pseudomonas* exotoxin A have been suggested as potential candidate toxins for this type of treatment. A  
15       difficulty of such methods has been the inability to selectively target specific cell types for the delivery of toxins or other active moieties.

One method of targeting specific cells has been to make fusion proteins of a toxin and a single chain antibody. A  
20       single-chain antibody (sFv) consists of an antibody light chain variable domain ( $V_L$ ) and heavy chain variable domain ( $V_H$ ), connected by a short peptide linker which allows the structure to assume a conformation capable of binding to antigen. In a diagnostic or therapeutic setting, the use of  
25       an sFv may offer attractive advantages over the use of a monoclonal antibody (MoAb). Such advantages include more rapid tumor penetration with concomitantly low retention in non-targeted organs (Yokota et al. *Cancer Res* 52:3402, 1992), extremely rapid plasma and whole body clearance (resulting in  
30       high tumor to normal tissue partitioning) in the course of imaging studies (Colcher et al. *Natl.Cancer Inst.* 82: 1191, 1990; Milenic et al. *Cancer Res.* 51:6363, 1991), and relatively low cost of production and ease of manipulation at  
35       the genetic level (Huston et al. *Methods Enzymol.* 203:46, 1991; Johnson, S. and Bird, R. E. *Methods Enzymol.* 203:88, 1991). In addition, sFv-toxin fusion proteins have been shown to exhibit enhanced anti-tumor activity in comparison with conventional chemically cross-linked conjugates (Chaudhary et

al. *Nature* 339:394, 1989; Batra et al. *Cell. Biol.* 11:2200-2295, 1991). Among the first sFv to be generated were molecules capable of binding haptens (Bird et al. *Science* 242:423, 1988; Huston et al. *Proc. Natl. Acad. Sci. USA* 85:5879, 1988), cell-surface receptors (Chaudhary et al., 1989), and tumor antigens (Chaudhary et al. *Proc. Natl. Acad. Sci. USA* 87:1066, 1990; Colcher et al., 1990).

The gene encoding an sFv can be assembled in one of two ways: (i) by *de novo* construction from chemically synthesized overlapping oligonucleotides, or (ii) by polymerase chain reaction (PCR)-based cloning of  $V_L$  and  $V_H$  genes from hybridoma cDNA. The main disadvantages of the first approach are the considerable expense involved in oligonucleotide synthesis, and the fact that the sequence of  $V_L$  and  $V_H$  must be known before gene assembly is possible. Consequently, the majority of the sFv reported to date were generated by cloning from hybridoma cDNA; nevertheless, this approach also has inherent disadvantages, because it requires availability of the parent hybridoma or myeloma cell line, and problems are often encountered when attempting to retrieve the correct V region genes from heterologous cDNA. For example, hybridomas in which the immortalizing fusion partner is derived from MOPC-21 may express a  $V_L$  kappa transcript which is aberrantly rearranged at the VJ recombination site, and which therefore encodes a non-functional light chain (Cabilly & Riggs, 1985; Carroll et al., 1988). Cellular levels of this transcript may exceed that generated from the productive  $V_L$  gene, so that a large proportion of the product on PCR amplification of hybridoma cDNA will not encode a functional light chain. A second disadvantage of the PCR-based method, frequently encountered by the inventors, is the variable success of recovering  $V_H$  genes using the conditions so far reported in the literature, presumably because the number of mismatches between primers and the target sequence destabilizes the hybrid to an extent which inhibits PCR amplification.

Thus, methods of targeting toxins to specific cells using single-chain antibodies methods have been difficult to

practice because of the difficulties in obtaining single chain antibodies and other targeting moieties. Also, none of the proposed treatment methods has been fully successful, because of the need to fuse the toxin to the targeting moiety, thus disrupting either the toxin function or the targeting function. Thus, a need exists for a means to target molecules having a desired activity to a specific cell population.

Bacterial and plant protein toxins have evolved novel and efficient strategies for penetrating to the cytosol of mammalian cells, and this ability has been exploited to develop anti-tumor and anti-HIV cytotoxic agents. Examples include ricin and *Pseudomonas exotoxin A* (PE) chimeric toxins and immunotoxins.

*Pseudomonas exotoxin A* (PE) is a toxin for which a detailed analysis of functional domains exists. The sequence is deposited with GenBank. Structural determination by X-ray diffraction, expression of deleted proteins, and extensive mutagenesis studies have defined three functional domains in PE: a receptor-binding domain (residues 1-252 and 365-399), designated Ia and Ib, a central translocation domain (amino acids 253-364, domain II), and a carboxyl-terminal enzymatic domain (amino acids 400-613, domain III). Domain III catalyzes the ADP-ribosylation of elongation factor 2 (EF-2), which results in inhibition of protein synthesis and cell death. Recently it was also found that an extreme carboxyl terminal sequence is essential for toxicity (Chaudhary et al. Proc. Natl. Acad. Sci. U.S.A. 87:308-312, 1990; Seetharam et al. J. Biol. Chem. 266:17376-17381, 1991). Since this sequence is similar to the sequence that specifies retention of proteins in the endoplasmic reticulum (ER) (Munro, S. and Pelham, H.R.B. Cell 48:899-907, 1987), it was suggested that PE must pass through the ER to gain access to the cytosol. Detailed knowledge of the structure of PE has facilitated use of domains II, Ib, and III (together designated PE40) in hybrid toxins and immunotoxins.

*Bacillus anthracis* produces three proteins which when combined appropriately form two potent toxins, collectively designated anthrax toxin. Protective antigen

(PA, 82,684 Da (Dalton) (SEQ ID NOS: 3 and 4)) and edema factor (EF, 89,840 Da) combine to form edema toxin (ET), while PA and lethal factor (LF, 90,237 Da (SEQ ID NOS: 1 and 2)) combine to form lethal toxin (LT) (Leppla, S.H. Alouf, J.E. 5 and Freer, J. H., eds. Academic Press, London 277-302, 1991). ET and LT each conform to the AB toxin model, with PA providing the target cell binding (B) function and EF or LF acting as the effector or catalytic (A) moieties. A unique feature of these toxins is that LF and EF have no toxicity in 10 the absence of PA, apparently because they cannot gain access to the cytosol of eukaryotic cells.

The genes for each of the three anthrax toxin components have been cloned and sequenced (Leppla, 1991). This showed that LF and EF have extensive homology in amino 15 acid residues 1-300. Since LF and EF compete for binding to PA63, it is highly likely that these amino-terminal regions are responsible for binding to PA63. Direct evidence for this was provided in a recent mutagenesis study (Quinn et al. *J. Biol. Chem.* 266:20124-20130, 1991); all mutations made within 20 amino acid residues 1-210 of LF led to decreased binding to PA63. The same study also suggested that the putative catalytic domain of LF included residues 491-776 (Quinn et al., 1991). In contrast, the location of functional domains within the PA63 polypeptide is not obvious from inspection of 25 the deduced amino acid sequence. However, studies with monoclonal antibodies and protease fragments (Leppla, 1991) and subsequent mutagenesis studies (Singh et al. *J. Biol. Chem.* 266:15493-15497, 1991) showed that residues at and near the carboxyl terminus of PA are involved in binding to 30 receptor.

PA is capable of binding to the surface of many types of cells. After PA binds to a specific receptor (Leppla, 1991) on the surface of susceptible cells, it is cleaved at a single site by a cell surface protease, probably 35 furin, to produce an amino-terminal 19-kDa fragment that is released from the receptor/PA complex (Singh et al. *J. Biol. Chem.* 264:19103-19107, 1989). Removal of this fragment from PA exposes a high-affinity binding site for LF and EF on the

receptor-bound 63-kDa carboxyl-terminal fragment (PA63). The complex of PA63 and LF or EF enters cells and probably passes through acidified endosomes to reach the cytosol.

Cleavage of PA occurs after residues 164-167, 5 Arg-Lys-Lys-Arg. This site is also susceptible to cleavage by trypsin and can be referred to as the trypsin cleavage site. Only after cleavage is PA able to bind either EF or LF to form either ET or LT.

Prior work had shown that the carboxyl terminal PA 10 fragment (PA63) can form ion conductive channels in artificial lipid membranes (Blaustein et al. Proc. Natl. Acad. Sci. U.S.A. 86:2209-2213, 1989; Koehler, T. M. and Collier, R.J. Mol. Microbiol. 5:1501-1506, 1991), and that LF bound to PA63 15 on cell surface receptors can be artificially translocated across the plasma membrane to the cytosol by acidification of the culture medium (Friedlander, A. M. J. Biol. Chem. 261:7123-7126, 1986). Furthermore, drugs that block endosome acidification protect cells from LF (Gordon et al. J. Biol. Chem. 264:14792-14796, 1989; Friedlander, 1986; Gordon et al. 20 Infect. Immun. 56:1066-1069, 1988). The mechanisms by which EF is internalized have been studied in cultured cells by measuring the increases in cAMP concentrations induced by PA and EF (Leppla, S. H. Proc. Natl. Acad. Sci. U.S.A. 79:3162- 3166, 1982; Gordon et al., 1989). However, because assays of 25 cAMP are relatively expensive and not highly precise, this is not a convenient method of analysis. Internalization of LF has been analyzed only in mouse and rat macrophages, because these are the only cell types lysed by the lethal toxin.

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#### SUMMARY OF THE INVENTION

The present invention provides a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the PA binding domain of the native LF protein and a nucleotide sequence encoding an activity inducing domain of a 35 second protein. Also provided is a nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein and a nucleotide sequence encoding a ligand domain

which specifically binds a cellular target. Proteins encoded by the nucleic acid of the invention, vectors comprising the nucleic acids and hosts capable of expressing the protein encoded by the nucleic acids are also provided.

5 A composition comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety is further provided.

10 A method for delivering an activity to a cell is provided. The steps of the method include administering to the cell (a) a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand domain and (b) a product comprising the PA binding domain of the native LF protein and a non-LF activity inducing moiety, whereby the product administered in step (b) is internalized 15 into the cell and performs the activity within the cell.

Characteristics unique to anthrax toxin are exploited to make novel cell-specific cytotoxins. A site in the PA protein of the toxin which must be proteolytically cleaved for the activity-inducing moiety of the toxin to enter 20 the cell is replaced by the consensus sequence recognized by a specific protease. Thus, the toxin will only act on cells infected with intracellular pathogens which make that specific protease.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of the percent to which mutant proteins are cleaved by purified HIV-1 protease. The mutant proteins include protective antigen (PA) mutated to include the HIV-1 protease cleavage site in place of the natural 30 trypsin cleavage site.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

##### Nucleic Acids

###### Lethal Factor (LF)

35 The present invention provides an isolated nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the PA binding domain of the native LF protein and a nucleotide sequence encoding an activity

inducing domain of a second protein. The LF gene and native LF protein are shown in SEQ ID NO: 1 and 2, respectively. The PA gene and native PA protein are shown in SEQ ID NO: 3 and 4, respectively.

5       The second protein can be a toxin, for example *Pseudomonas exotoxin A* (PE), the A chain of *Diphtheria* toxin or shiga toxin. The activity inducing domains of numerous other known toxins can be included in the fusion protein encoded by the presently claimed nucleic acid. The activity  
10      inducing domain need not be a toxin, but can have other activities, including but not limited to stimulating or reducing growth, selectively inhibiting DNA replication, providing a desired gene, providing enzymatic activity or providing a source of radiation. In any case, the fusion  
15      proteins encoded by the nucleic acids of the present invention must be capable of being internalized and capable of expressing the specified activity in a cell. A given LF fusion protein of the present invention can be tested for its ability to be internalized and to express the desired activity  
20      using methods as described herein, particularly in Examples 1 and 2.

An example of a nucleic acid of the invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 5. This nucleic acid encodes a fusion of LF residues 1-254 with the two-residue linker "TR" and PE residues 401-602 (SEQ ID NO: 6). The protein includes a Met-Val-Pro- sequence at the beginning of the LF sequence. Means for obtaining this fusion protein are further described below and in Example 1.

30       A further example of a nucleic acid of this invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 7. This nucleic acid encodes a fusion of LF residues 1-254 with the two-residue linker "TR" and PE residues 398-613. (SEQ ID NO: 8) The junction point  
35      containing the "TR" is the sequence LTRA and the Met-Val-Pro- is also present. This fusion protein and methods for obtaining it are further described below and in Example 2.

Another example of the nucleic acid of the present invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO: 9. This nucleic acid encodes a fusion of LF residues 1-254 with the two residue linker and PE residues 362-613. (SEQ ID NO: 10) This fusion protein is further described in Example 1.

Alternatively, the nucleic acid can include the entire coding sequence for the LF protein fused to a non-LF activity inducing domain. Other LF fusion proteins of various sizes and methods of making and testing them for the desired activity are also provided herein, particularly in Examples 1 and 2.

Protective Antigen (PA)

Also provided is an isolated nucleic acid encoding a fusion protein comprising a nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target.

An example of a nucleic acid of this invention comprises the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:11. This nucleic acid encodes a fusion of PA residues 1-725 and human CD4 residues 1-178, the portion which binds to gp120 exposed on HIV-1 infected cells (SEQ ID NO:12). This fusion protein and methods for obtaining and testing fusion proteins are further described below and in Examples 3, 4 and 5.

The PA fusion protein encoding nucleic acid provided can encode any ligand domain that specifically binds a cellular target, e.g. a cell surface receptor, an antigen expressed on the cell surface, etc. For example, the nucleic acid can encode a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell. Such a ligand domain can be a single chain antibody which is expressed as a fusion protein as provided above and in Examples 3, 4 and 5. Alternatively, the nucleic acid can encode, for example, a ligand domain that is a growth factor, as provided in Example 3.

Although the PA encoding sequence of the nucleic acid encoding the PA fusion proteins of this invention need only include the nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein, the nucleic acid can further comprise the nucleotide sequence encoding the remainder of the native PA protein. Any sequences to be included beyond those required, can be determined based on routine considerations such as ease of manipulation of the nucleic acid, ease of expression of the product in the host, and any effect on translocation/internalization as taught in the examples.

#### Proteins

Proteins encoded by the nucleic acids of the present invention are also provided.

#### LF Fusion Proteins

The present invention provides LF fusion proteins encoded by the nucleic acids of the invention as described above and in the examples. Specifically, fusions of the LF gene with domains II, Ib, and III of PE can be made by recombinant methods to produce in-frame translational fusions. Recombinant genes (e.g., SEQ ID NOs: 5, 7 and 9) were expressed in *Escherichia coli* (*E. coli*), and the purified proteins were tested for activity on cultured cells as provided in Examples 1 and 2. Certain fusion proteins are efficiently internalized via the PA receptor to the cytosol. These examples demonstrate that this system can be used to deliver many different polypeptides into targeted cells.

Although specific examples of these proteins are provided, given the present teachings regarding the preparation of LF fusion proteins, other embodiments having other activity inducing domains can be practiced using routine skill.

Using current methods of genetic manipulation, a variety of other activity inducing moieties (e.g., polypeptides) can be translated as fusion proteins with LF which in turn can be internalized by cells when administered with PA or PA fusion proteins. Fusion proteins generated by

this method can be screened for the desired activity using the methods set forth in the Examples and by various routine procedures. Based on the data presented here, the present invention provides a highly effective system for delivery of 5 an activity inducing moiety into cells.

PA fusion proteins

The present invention provides PA fusion proteins encoded by the nucleic acids of the invention. Specifically fusions of PA with single chain antibodies and CD4 are 10 provided.

Using current methods of genetic manipulation, a variety of other ligand domains (e.g., polypeptides) can be translated as fusion proteins with PA which in turn can specifically target cells and facilitate internalization LF or 15 LF fusion proteins. Based on the data presented here, the present invention provides a highly effective system for delivery of an activity inducing moiety into a particular type or class of cells.

Although specific examples of these proteins are 20 provided, given the present teachings regarding the preparation of PA fusion proteins, other embodiments having other ligand domains can be practiced using routine skill. The fusion proteins generated can be screened for the desired specificity and activity utilizing the methods set forth in 25 the example and by various routine procedures. In any case, the PA fusion proteins encoded by the nucleic acids of the present invention must be able to specifically bind the selected target cell, bind LF or LF fusions or conjugates and internalize the LF fusion/conjugate.

30 Conjugates

A composition comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety is provided. Such an activity inducing moiety is an activity not present on native LF. The composition can 35 comprise an activity inducing moiety that is, for example, a polypeptide, a radioisotope, an antisense nucleic acid or a nucleic acid encoding a desired gene product.

Using current methods of chemical manipulation, a variety of other moieties (e.g., polypeptides, nucleic acids, radioisotopes, etc.) can be chemically attached to LF and can be internalized into cells and can express their activity when administered with PA or PA fusion proteins. The compounds can be tested for the desired activity and internalization following the methods set forth in the Examples. For example, the present invention provides an LF protein fragment 1-254 (LF1-254) with a cysteine residue added at the end of LF1-254 (LF1-254Cys). Since there are no other cysteines in LF, this single cysteine provides a convenient attachment point through which to chemically conjugate other proteins or non-protein moieties.

#### Vectors and Hosts

A vector comprising the nucleic acids of the present invention is also provided. The vectors of the invention can be in a host capable of expressing the protein encoded by the nucleic acid.

To express the proteins and conjugates of the present invention, the nucleic acids can be operably linked to signals that direct gene expression. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The gene encoding a protein of the invention can be inserted into an "expression vector", "cloning vector", or "vector," terms which usually refer to plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors can replicate autonomously, or they can replicate by being inserted into the genome of the host cell. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is

desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

The particular vector used to transport the genetic information into the cell is also not particularly critical. Any of the conventional vectors used for expression of recombinant proteins in prokaryotic or eukaryotic cells can be used.

The expression vectors typically have a transcription unit or expression cassette that contains all the elements required for the expression of the DNA encoding a protein of the invention in the host cells. A typical expression cassette contains a promoter operably linked to the DNA sequence encoding the protein, and signals required for efficient polyadenylation of the transcript. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

The DNA sequence encoding the protein of the invention can be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Additional elements of the vector can include, for example, selectable markers and enhancers. Selectable markers, e.g., tetracycline resistance or hygromycin resistance, permit detection and/or selection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus, the long terminal repeat from various retroviruses such as murine leukemia virus, murine or

Rous sarcoma virus, and HIV. See, *Enhancers and Eukaryotic Expression*, Cold Spring Harbor Pres, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region can be obtained from the same gene as the promoter sequence or can be obtained from a different gene.

For more efficient translation in mammalian cells of the mRNA encoded by the structural gene, polyadenylation sequences are also commonly added to the vector construct. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40, or a partial genomic copy of a gene already resident on the expression vector.

The vectors containing the gene encoding the protein of the invention are transformed into host cells for expression. "Transformation" refers to the introduction of vectors containing the nucleic acids of interest directly into host cells by well known methods. The particular procedure used to introduce the genetic material into the host cell for expression of the protein is not particularly critical. Any of the well known procedures for introducing foreign nucleotide sequences into host cells can be used. It is only necessary that the particular procedure utilized be capable of successfully introducing at least one gene into the host cell which is capable of expressing the gene.

Transformation methods, which vary depending on the type of host cell, include electroporation; transfection employing calcium chloride, rubidium chloride calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent); and other methods. See, generally,

Sambrook et al., (1989) *supra*, and *Current Protocols in Molecular Biology*, *supra*. Reference to cells into which the nucleic acids described above have been introduced is meant to also include the progeny of such cells.

5 There are numerous prokaryotic expression systems known to one of ordinary skill in the art useful for the expression of the antigen. *E. coli* is commonly used, and other microbial hosts suitable for use include *bacilli*, such as *Bacillus subtilis*, and other enterobacteriaceae, such as  
10 *Salmonella*, *Serratia*, and various *Pseudomonas* species. One can make expression vectors for use in these prokaryotic hosts; the vectors will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication, a promoter). Any number of a variety of well-known promoters can be used, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site  
15 sequences, for example, for initiating and completing transcription and translation. If necessary, an amino terminal methionine can be provided by insertion of a Met codon 5' and in-frame with the codons for the protein. Also, the carboxy-terminal end of the protein can be removed using  
20 standard oligonucleotide mutagenesis procedures, if desired.  
25

Host bacterial cells can be chosen that are mutated to be reduced in or free of proteases, so that the proteins produced are not degraded. For *Bacillus* expression systems in which the proteins are secreted into the culture medium, strains are available that are deficient in secreted  
30 proteases.

Mammalian cell lines can also be used as host cells for the expression of polypeptides of the invention. Propagation of mammalian cells in culture is *per se* well known. See, *Tissue Culture*, Academic Press, Kruse and Patterson, ed. (1973). Host cell lines may also include such organisms as bacteria (e.g., *E. coli* or *B. subtilis*), yeast, filamentous fungi, plant cells, or insect cells, among others.

Purification of Protein

After standard transfection or transformation methods are used to produce prokaryotic, mammalian, yeast, or insect cell lines that express large quantities of the protein of the invention, the protein is then purified using standard techniques which are known in the art. See, e.g., Colley et al. (1989) *J. Biol. Chem.* 64: 17619-17622; and *Methods in Enzymology*, "Guide to Protein Purification", M. Deutscher, ed. Vol. 182 (1990).

Standard procedures of the art that can be used to purify proteins of the invention include ammonium sulfate precipitation, affinity and fraction column chromatography, gel electrophoresis and the like. See, generally, Scopes, R., *Protein Purification*, Springer-Verlag, New York (1982), and U.S. Pat. No. 4,512,922 disclosing general methods for purifying protein from recombinantly engineered bacteria.

If the expression system causes the protein of the invention to be secreted from the cells, the recombinant cells are grown and the protein is expressed, after which the culture medium is harvested for purification of the secreted protein. The medium is typically clarified by centrifugation or filtration to remove cells and cell debris and the proteins can be concentrated by adsorption to any suitable resin such as, for example, CDP-Sepharose, asialoprothrombin-Sepharose 4B, or Q Sepharose, or by use of ammonium sulfate fractionation, polyethylene glycol precipitation, or by ultrafiltration. Other means known in the art are equally suitable. Further purification of the protein can be accomplished by standard techniques, for example, affinity chromatography, ion exchange chromatography, sizing chromatography, or other protein purification techniques used to obtain homogeneity. The purified proteins are then used to produce pharmaceutical compositions, as described below.

Alternatively, vectors can be employed that express the protein intracellularly, rather than secreting the protein from the cells. In these cases, the cells are harvested, disrupted, and the protein is purified from the cellular extract, e.g., by standard methods. If the cell line has a

cell wall, then initial extraction in a low salt buffer may allow the protein to pellet with the cell wall fraction. The protein can be eluted from the cell wall with high salt concentrations and dialyzed. If the cell line glycosolates the protein, then the purified glycoprotein may be enhanced by using a Con A column. Anion exchange columns (MonoQ, Pharmacia) and gel filtration columns may be used to further purify the protein. A highly purified preparation can be achieved at the expense of activity by denaturing preparative polyacrylamide gel electrophoresis.

Protein analogs can be produced in multiple conformational forms which are detectable under nonreducing chromatographic conditions. Removal of those species having a low specific activity is desirable and is achieved by a variety of chromatographic techniques including anion exchange or size exclusion chromatography.

Recombinant analogs can be concentrated by pressure dialysis and buffer exchanged directly into volatile buffers (e.g., N-ethylmorpholine (NEM), ammonium bicarbonate, ammonium acetate, and pyridine acetate). In addition, samples can be directly freeze-dried from such volatile buffers resulting in a stable protein powder devoid of salt and detergents. In addition, freeze-dried samples of recombinant analogs can be efficiently resolubilized before use in buffers compatible with infusion (e.g., phosphate buffered saline). Other suitable buffers might include hydrochloride, hydrobromide, sulphate acetate, benzoate, malate, citrate, glycine, glutamate, and aspartate.

30

#### Specific Embodiments

##### Toxins Modified to Contain Intracellular Pathogen Protease Recognition sites

One aspect of the invention exploits the fact that PA and other toxins must be proteolytically cleaved in order to acquire activity, in conjunction with the fact that some cells infected with an intracellular pathogen possess an active protease that has a relatively narrow substrate specificity (for example, HIV-infected cells). The protease

site found in the native toxin is replaced with an intracellular pathogen specific protease site. Thus, the protease in cells that are infected by the intracellular pathogen cleaves the modified toxin, which then becomes active and kills the cell.

Intracellular pathogens that can be targeted by the products and methods of the present invention include any pathogen that produces a protease having a specific recognition site. Such pathogens can include prokaryotes (including rickettsia, *Mycobacterium tuberculosis*, etc.), mycoplasma, eukaryotic pathogens (e.g. pathogenic fungi, etc.), and viruses. One example of an intracellular pathogen that produces a specific protease is human immunodeficiency virus (HIV). The HIV-1 protease cleaves viral polyproteins to generate functional structural proteins as well as the reverse transcriptase and the protease itself. HIV-1 replication and viral infectivity are absolutely dependent on the action of the HIV-1 protease.

An intracellular pathogen specific protease site can be introduced into any natural or recombinant toxin for which proteolytic cleavage is required for toxicity. For example, one can replace the anthrax PA trypsin cleavage site (R164-167) of PA with the HIV-1 protease site. Alternatively, the diphtheria toxin disulfide loop sequence (see O'Hare, et al. 20 FEBS 273 (1, 2): 200-204 (Oct. 1990)) can be replaced with the HIV-1 protease cleavage site in order to obtain a toxin specific to HIV-1 infected cells. Similarly, the normally occurring diphtheria toxin sequence at residues 191-194 (Williams, et al. J. Biol. Chem. 265(33): 20673-20677 (1990)) 25 can be replaced by an intracellular pathogen specific protease site such as the HIV-1 protease cleavage sequence. The DAB486-IL-2 fusion toxin of Williams and the improved DAB389-IL-2 toxin are effective on HIV-1 infected cells, which express high levels of the IL-2 receptor. Williams, J. Biol. Chem. 265:20673. Addition of the HIV-1 protease cleavage site 30 would provide a further degree of specificity. Similarly, the botulinum toxin C2 toxin is like the anthrax toxin in requiring a cleavage within a native protein subunit (see

Ohishi and Yanagimoto, *Infection and Immunity* 60(11): 4648-4655 (Nov. 1992)), so it too can be made specific for cells infected by an intracellular pathogen such as HIV-1.

In one embodiment of the invention, the protease site of PA is replaced by the site recognized by the HIV-1 protease. The cellular protease that cleaves PA absolutely requires the presence of the Arg 164 and Arg 167 residues, because replacement of either residue yields a PA molecule which is not cleaved after binding to the cell surface.

However, any PA substitution mutant which retains at least one Arg or Lys residue within residues 164-167 can be activated by treatment with trypsin. Because the PA63 fragments produced by trypsin digestion have a variety of different amino terminal residues, it is clear that there is not a strict constraint on the identity of the terminal residues. Klimpel, et al., *Proc. Natl. Acad. Sci.* 89:10277-10281 (1992).

Replacement of residues 164-167 of PA with residues that match the HIV-1 protease recognition site can render exogenously added PA inactive on cells which do not possess the HIV-1 protease. However, those cells that do express the HIV-1 protease (i.e., cells infected with HIV-1 or cells engineered to produce the protease) would cleave and thereby activate the mutant PA. The activated PA proteins can then bind and internalize cytotoxic fusion proteins, such as LF-PE, added exogenously.

Based on extensive studies of the substrate specificity of the protease, several PA variants were designed and produced which relate to the invention. These are shown below, with the residues underlined between which the cleavage occurred. PA proteins which have been mutated to replace R164-167 with an amino acid sequence recognized by the HIV-1 protease are referred to as "PAHIV."

PAHIV#1	QVSQNYPIVQNI
PAHIV#2	NTATIM <u>M</u> QRGNF
PAHIV#3	TVSFNF <u>P</u> QITLW
PAHIV#4	GGS <u>A</u> FN <u>F</u> PIVMGG

The mutant proteins PAHIV#(1-4) were cleaved correctly by the HIV-1 protease.

Table 1 shows the amino acids and their corresponding abbreviations and symbols.

Table 1

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

15 Preferably, the mutations at R164-167 of PA are accomplished by cassette mutagenesis, although other methods are feasible as discussed below. In summary, three pieces of DNA are joined together. The first piece has vector sequences and encodes the "front half" (5' end of the gene) of PA protein, the second is a short piece of DNA (a cassette) and encodes a small middle piece of PA protein and the third encodes the "back half" (3' end of the gene) of PA. The cassette contains codons for the amino acids that are required to complete the cleavage site for the intracellular pathogen protease. This method was used to make mutants in the plasmid pYS5 although other plasmids could be employed.

20 Alternatively, the mutations can be accomplished by use of the polymerase chain reaction (PCR) and other methods as discussed below. PCR duplicates a segment of DNA many times, resulting in an amplification of that segment. The reaction produces enough of the segment of DNA so that it can be modified with restriction enzymes and cloned. During the reaction a synthetic oligonucleotide primer is used to start the duplication of the target DNA segment. Each synthetic

primer can be designed to introduce novel DNA sequences into the DNA molecule, or to change existing DNA sequences.

Modification of Toxins to Broaden or Alter Target Cell

5      Specificity

Another aspect of the invention involves compounds and methods for broadening or changing the range of cell types against which a toxin is effective. For example, the lethal anthrax toxin, PA+LF, is acutely toxic to mouse macrophage 10 cells, apparently due to the specific expression in these cells of a target for the catalytic activity of LF. Other cell types are not affected by LF. However, in the present invention, LF is used to construct cytotoxins having broad cell specificity.

15      A detailed analysis of the domains of LF identified the amino-terminal 254 amino acids as the region that binds to PA63. Fusion proteins containing residues 1-254 of LF and the ADP-ribosylation domain of *Pseudomonas* exotoxin A (PE) were designed according to the invention. These fusion proteins 20 are highly toxic to cultured cells, but only when PA is administered simultaneously.

Synthesis of Genes that Encode Proteins of the Invention

Genes that encode toxins having altered protease 25 recognition sites or fusion proteins having a binding domain from one protein and an activity inducing domain of a second protein can be synthesized by methods known to those skilled in the art. As an example of techniques that can be utilized, the synthesis of genes encoding modified anthrax toxin subunits LF and PA are now described.

30      The DNA sequences for native PA and LF are known. Knowledge of these DNA sequences facilitates the preparation of genes and can be used as a starting point to construct DNA molecules that encode mutants of PA and/or LF. The protein mutants of the invention are soluble and include internal 35 amino acid substitutions. Furthermore, these mutants are purified from, or secreted from, cells that have been transfected or transformed with plasmids containing genes which encode these proteins. Methods for making

modifications, such as amino acid substitutions, deletions, or the addition of signal sequences to cloned genes are known. Specific methods used herein are described below.

The gene for PA or LF can be prepared by several methods. Genomic and cDNA libraries are commercially available. Oligonucleotide probes, specific to the desired gene, can be synthesized using the known gene sequence. Methods for screening genomic and cDNA libraries with oligonucleotide probes are known. A genomic or cDNA clone can provide the necessary starting material to construct an expression plasmid for the desired protein using known methods.

A protein encoding DNA fragment can be cloned by taking advantage of restriction endonuclease sites which have been identified in regions which flank or are internal to the gene. See Sambrook, et al., *Molecular Cloning: A Laboratory Manual* 2d.ed. Cold Spring Harbor Laboratory Press (1989), "Sambrook" hereinafter.

Genes encoding the desired protein can be made from wild-type genes constructed using the gene encoding the full length protein. One method for producing wild-type genes for subsequent mutation combines the use of synthetic oligonucleotide primers with polymerase extension on a mRNA or DNA template. This PCR method amplifies the desired nucleotide sequence. U.S. Patents 4,683,195 and 4,683,202 describe this method. Restriction endonuclease sites can be incorporated into the primers. Genes amplified by PCR can be purified from agarose gels and cloned into an appropriate vector. Alterations in the natural gene sequence can be introduced by techniques such as *in vitro* mutagenesis and PCR using primers that have been designed to incorporate appropriate mutations.

The proteins described herein can be expressed intracellularly and purified, or can be secreted when expressed in cell culture. If desired, secretion can be obtained by the use of the native signal sequence of the gene. Alternatively, genes encoding the proteins of the invention can be ligated in proper reading frame to a signal sequence

other than that corresponding to the native gene. Though the PA recombinant proteins of the invention are typically expressed in *B. anthracis*, they can be expressed in other hosts, such as *E. coli*.

5       The proteins of this invention are described by their amino acid sequences and by their nucleotide sequence, it being understood that the proteins include their biological equivalents such that this invention includes minor or inadvertent substitutions and deletions of amino acids that  
10      have substantially little impact on the biological properties of the analogs. In some circumstances it may be feasible to substitute rare or non-naturally occurring amino acids for one or more of the twenty common amino acids listed in Table 2. Examples include ornithine and acetylated or hydroxylated  
15      forms. See generally Stryer, L., *Biochemistry* 3d ed. (1988).

Alternative nucleotide sequences can be used to express analogs in various host cells. Furthermore, due to the degeneracy of the genetic code, equivalent codons can be substituted to encode the same polypeptide sequence.

20      Additionally, sequences (nucleotide and amino acid) with substantial identity to those of the invention are also included. Identity in this sense means the same identity (of base pair or amino acid) and order (of base pairs or amino acids). Substantial identity includes entities that are  
25      greater than 80% identical. Preferably, substantial identity refers to greater than 90% identity. More preferably, it refers to greater than 95% identity.

#### Mutagenesis

30      Mutagenesis can be performed to yield point mutations, deletions, or insertions to alter the specific regions of the genes described above. Point mutations can be introduced by a variety of methods including chemical mutagenesis, mutagenic copying methods and site specific mutagenesis methods using  
35      synthetic oligonucleotides.

Cassette mutagenesis methods are conveniently used to introduce point mutations into the specified regions of the PA or LF genes. A double-stranded oligonucleotide region

containing alterations in the specified sequences of the gene is prepared. This oligonucleotide cassette region can be prepared by synthesizing an oligonucleotide with the sequence alteration in residues of the PA or LF gene, annealing to a 5 primer, elongating with the large fragment of DNA polymerase and trimming with BstBI. This double-stranded oligonucleotide is ligated into the BamHI/BstBI fragment from pYS5 and the PpuMI-BamHI fragment from pYS6 to produce an intact recombinant DNA. Other methods of producing the double 10 stranded oligonucleotides and other recombinant DNA vectors can be practiced.

Chemical mutagenesis can be performed using the M13 vector system. A single strand M13 recombinant DNA is prepared containing recombinant PA or LF DNA. Another M13 15 recombinant containing the same recombinant DNA but in double stranded form is used to prepare a deletion in the targeted region of the gene. This double stranded M13 recombinant is cleaved into a linear molecule with an endonuclease, denatured, and annealed with the single strand M13 20 recombinant, resulting in a single strand gap in the target region of the PA or LF DNA.

This gapped DNA M13 recombinant is then treated with a compound such as sodium bisulfite to deaminate the cytosine residues in the single strand DNA region to uracil. This 25 results in limited and specific mutations in the single strand DNA region. Finally, the gap in the DNA is filled in by incubation with DNA polymerase, resulting in a U-A base pair to replace a G-C base pair in the unmutated portion of the gene. Upon replication the new recombinant gene contains T-A 30 base pairs, which are point mutations from the original sequence. Other forms of chemical mutagenesis are also available.

Mutagenic copying of the PA or LF recombinant DNA can be carried out using several methods. For example, a single-stranded gapped DNA region is created as described above. 35 This region is incubated with DNA polymerase I and one or more mutagenic analogs of normal ribonucleoside triphosphates. Copying of the single stranded region with the DNA polymerase

substitutes the mutagenic analogs as the single strand gap region is filled in. Transfection and replication of the resulting DNA results in production of some mutated recombinant DNAs for PA, LF, or EF which can then be selected by cloning. Other mutagenic copying methods can be used.

Point mutations can be introduced into the specified regions of the PA or LF genes by methods using synthetic oligonucleotides for site-specific mutagenesis. PCR copying of the PA or LF genes is performed using oligonucleotide primers covering the specified target regions, and which contain modifications from the wild type sequence in these regions. The PA gene in a pYS5 vector can be PCR amplified using this method to result in mutations in the 164-167 position. PCR amplification can also be used to introduce mutations in the target region of the LF gene.

Synthetic oligonucleotide methods of introducing point mutations can be preformed using heteroduplex DNA. A M13 recombinant DNA vector containing the PA or LF gene is prepared and a single-stranded M13 recombinant is produced. A single strand oligonucleotide containing an alteration in the specified target sequence for the PA or LF gene is annealed to the single strand M13 recombinant to produce a mismatched sequence. Incubation with DNA polymerase I results in a double-stranded M13 recombinant containing base pair mismatches in the specified region of the gene. This M13 recombinant is replicated in a host such as *B. anthracis* or *E. coli* to produce both wild type and mutant M13 recombinants. The mutated M13 recombinants are cloned and isolated. Other vector systems for mutagenesis involving synthetic nucleotides and heteroduplex formation can be applicable.

#### Expression of Proteins in Prokaryotic Cells

In addition to the use of cloning methods in bacteria such as *Bacillus anthracis* for amplification of cloned sequences, it may be desirable to express the proteins in other prokaryotes. It is possible to recover a functional protein from *E. coli* transformed with an expression plasmid encoding a PA or LF protein. Conveniently, the mutated PA

proteins of the invention were expressed in *B. anthracis* and the LF-fusion proteins were expressed in *E. coli*.

Methods for the expression of cloned genes in bacteria are well known. See Sambrook. To optimize expression of a 5 cloned gene in a prokaryotic system, expression vectors can be constructed which include a promoter to direct mRNA transcription termination. The inclusion of selection markers in DNA vectors transformed in bacteria are useful. Examples of such markers include the genes specifying resistance to 10 ampicillin, tetracycline, or chloramphenicol.

See Sambrook, previously cited, for details concerning 15 selection markers and promoters for use in bacteria such as *E. coli*. In an embodiment of this invention, pYS5 is a vector for the subcloning and amplification of desired gene sequences although other vectors could be used.

Strains of *Bacillus anthracis* producing mutated protein(s)

For PA protein production, *B. anthracis* strains cured 20 of both pX01 and pX02 are preferred because they are avirulent. Examples of such strains are UM23Cl-1 and UM44-1C9, obtained from Curtis Thorne, University of Massachusetts. Similar strains can be made by curing of plasmids, as described by P. Mikesell, et al., "Evidence for 25 plasmid-mediated toxin production in *Bacillus anthracis*," *Infect. Immun.* 39:371-376 (1983).

See generally commonly assigned U.S. Patent Application Serial No. 08/042,745, filed April 5, 1993, incorporated by reference herein.

30 Treatment Methods

A method for delivering a desired activity to a cell is provided. The steps of the method include administering to the cell (a) a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand 35 domain, and (b) a product comprising the PA binding domain of the native LF protein and a non-LF activity inducing moiety, whereby the product administered in step (b) is internalized into the cell and performs the activity within the cell.

The method of delivering an activity to a cell can use a ligand domain that is the receptor binding domain of the native PA protein. Other ligand domains are selected for their specificity for a particular cell type or class of 5 cells. The specificity of the PA fusion protein for the targeted cell can be determined using standard methods and as described in Examples 2 and 3.

The method of delivering an activity to a cell can use an activity inducing moiety that is a polypeptide, for example 10 a growth factor, a toxin, an antisense nucleic acid, or a nucleic acid encoding a desired gene product. The actual activity inducing moiety used will be selected based on its functional characteristics, e.g. its activity.

A method of killing a tumor cell in a subject is also 15 provided. The steps of the method can include administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a tumor cell specific ligand domain in an amount sufficient to bind to a tumor cell. A second fusion protein 20 is also administered wherein the protein comprises the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the tumor cell and kills the tumor cell.

25 The cytotoxic domain can be a toxin or it can be another moiety not strictly defined as a toxin, but which has an activity that results in cell death. These cytotoxic moieties can be selected using standard tests of cytotoxicity, such as the cell lysis and protein synthesis inhibition assays 30 described in the examples.

The invention further provides a method of killing HIV-infected cells in a subject. The method comprises the steps of administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of 35 the native PA protein and a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell, in an amount sufficient to bind to an HIV-infected cell. The next step is administering to the subject

a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein, in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the HIV-infected cell and kills the HIV-infected cell, thereby preventing propagation of HIV.

Although certain of the methods of the invention have been described as using LF fusion proteins, it will be understood that other LF compositions having chemically attached activity inducing moieties can be used in the methods.

The fusion proteins and other compositions of the inventions can be administered by various methods, e.g., parenterally, intramuscularly or intraperitoneally.

The amount necessary can be deduced from other receptor/ligand or antibody/antigen therapies. The amount can be optimized by routine procedures. The exact amount of such LF and PA compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the disease that is being treated, the particular fusion protein of composition used, its mode of administration, and the like. Generally, dosage will approximate that which is typical for the administration of cell surface receptor ligands, and will preferably be in the range of about 2 µg/kg/day to 2 mg/kg/day.

Depending on the intended mode of administration, the compounds of the present invention can be in various pharmaceutical compositions. The compositions will include, as noted above, an effective amount of the selected protein in combination with a pharmaceutically acceptable carrier and, in addition, can include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material can be administered to an individual along with the fusion protein or other composition without causing any undesirable biological effects or interacting in a deleterious manner with

any of the other components of the pharmaceutical composition in which it is contained.

Parenteral administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,710,795, which is incorporated by reference herein.

#### Formulations and Administration

Proteins of the invention such as PAHIV are typically mixed with a physiologically acceptable fluid prior to administration to a mammal such as a human. Examples of physiologically acceptable fluids include saline solutions such as normal saline, Ringer's solution, and generally mixtures of various salts including potassium and phosphate salts with or without sugar additives such as glucose. The proteins are administered parenterally with intravenous administration being the most typical route. Either a bolus of the protein in solution or a slow infusion can be administered intravenously. The choice of a bolus or an infusion depends on the kinetics, including the half-life, of the protein in the patient. An appropriate evaluation of the time for delivery of the protein is well within the skill of the clinician.

Patients selected for treatment with PAHIV are infected with HIV-1 and they may or may not be symptomatic. Optimally, the protein would be administered to an HIV-1 infected person who is not yet symptomatic. The dosage range of a protein of the invention such as PAHIV is typically from about 5 to about 25 micrograms per kilogram of body weight of the patient. Usually, the dose is about 10 micrograms per kilogram of body weight of the patient. The dosage is repeated at regular intervals, such as weekly for about 4 to 6 weeks. At that time the clinician may opt to evaluate the

patient's immune status, including immuno-tolerance to the PAHIV, to decide future treatment.

The foregoing description and the following examples are offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the operating conditions, materials, procedural steps and other parameters of the system described herein can be further modified or substituted in various ways without departing from the spirit and scope of the invention. For example, although human use has been discussed, veterinary use of the invention is also feasible. For instance, cats suffer from a so-called feline AIDS or feline immunodeficiency virus (FIV). Protective antigen can be altered to include a protease cleavage site specific for FIV. Thus, the invention is not limited by the description and examples, but rather by the appended claims.

EXAMPLE 1

Fusions of Anthrax Toxin Lethal Factor to the  
ADP-Ribosylation Domain of Pseudomonas Exotoxin  
Reagents and General Procedures

Restriction endonucleases and DNA modifying enzymes were purchased from GIBCO/BRL, Boehringer Mannheim, or New England Biolabs. Low melting point agarose (Sea Plaque) was obtained from FMC Corp. (Rockland, ME). Oligonucleotides were synthesized on a PCR Mate (Applied Biosystems) and purified on oligonucleotide purification cartridges (Applied Biosystems). The PCR was performed with a DNA amplification reagent (GeneAmp) from Perkin-Elmer Cetus Instruments and a thermal cycler (Perkin-Elmer Cetus). The amplification involved denaturation at 94°C for 1 min, annealing at 55°C for 2.5 min and extension at 72°C for 3 min, for 30 cycles. A final extension was run at 72°C for 7 min. For amplification of PE fragments, 10% formamide was added in the reaction mixture to decrease the effect of high GC content. DNA sequencing reactions were done using the Sequenase version 1.0 from U. S. Biochemical Corp. and DNA sequencing gels were made from Gel Mix 6 from GIBCO/BRL. [<sup>35</sup>S]deoxyadenosine 5'-[ $\alpha$ -

thio]triphosphate and L-[3,4,5-<sup>3</sup>H]leucine were purchased from Dupont-New England Nuclear. J774A.1 cells were obtained from American Type Culture Collection. Chinese Hamster Ovary (CHO) cells were obtained from Michael Gottesman (National Cancer Institute, National Institutes of Health) (ATCC CCL 61).

5 Plasmid Construction

Construction of plasmids containing LF-PE fusions was performed as follows. Varying portions of the PE gene were amplified by PCR, ligated in frame to the 3' end of the LF gene, and inserted into the pVEX115 f+T expression vector (provided by V. K. Chaudhary, National Cancer Institute, National Institutes of Health). To construct fusion proteins, the 3'-end of the native LF gene (including codon 776 of the mature protein, specifying Ser) was ligated with the 5'-ends 10 of sequences specifying varying portions of domains II, Ib, and III of PE. The LF gene was amplified from the plasmid pLF7 (Robertson, D. L. and Leppla, S.H. Gene 44:71-78, 1986) by PCR using oligonucleotide primers which added *Kpn*I and *Mlu*I sites at the 5' and the 3' ends of the gene, respectively.

15 Similarly, varying portions of the PE gene (provided by David FitzGerald, National Cancer Institute, National Institutes of Health) were amplified by PCR so as to add *Mlu*I and *Eco*RI sites at the 5' and 3' ends. The PCR product of the LF gene was digested with *Kpn*I and the DNA was precipitated. The LF gene was subsequently treated with *Mlu*I. Similarly, the PCR products of PE amplification were digested with *Mlu*I and *Eco*RI. The expression vector pVEX115 f+T was cleaved with *Kpn*I and *Eco*RI separately and dephosphorylated. This vector has a T7 promoter, OmpA signal sequence, multiple cloning site, and T7 transcription terminator. All the above DNA 20 fragments were purified from low-melting point agarose, a three-fragment ligation was carried out, and the product transformed into *E. coli* DH5 $\alpha$  (ATCC 53868). The four constructs described in this report have the entire LF gene 25 fused to varying portions of PE. The identity of each construct was confirmed by sequencing the junction point using a Sequenase kit (U.S. Biochemical Corp.). For expression, recombinant plasmids were transformed into *E. coli* strain 30

SA2821 (provided by Sankar Adhya, National Cancer Institute, National Institutes of Health, which is a derivative of BL21(λDE3) (Studier, F. W. and Moffatt, B.A. *J. Mol. Biol.* 189:113-150, 1986). This strain has the T7 RNA polymerase gene under control of an inducible lac promotor and also contains the *degP* mutation, which eliminates a major periplasmic protease (Strauch et al. *J. Bacteriol.* 171:2689-2696, 1989).

In the resulting plasmids, the LF-PE fusion genes are under control of the T7 promoter and contain an OmpA signal peptide to obtain secretion of the products to the periplasm so as to facilitate purification. The design of the PCR linkers also led to insertion of two non-native amino acids, Thr-Arg, at the LF-PE junction. The four fusions analyzed in this report contain the entire 776 amino acids of mature LF, the two added residues TR (Thr-Arg), and varying portions of PE. In fusion FP33, the carboxyl-terminal end of PE was changed from the native REDLK (Arg-Glu-Asp-Leu-Lys) to LDER, a sequence that fails to cause retention in the ER (endoplasmic reticulum).

#### Expression and Purification of Fusion Proteins

Fusion proteins produced from pNA2, pNA4, pNA23 and pNA33 were designated FP2, FP4, FP23 and FP33 respectively. *E. coli* strains carrying the recombinant plasmids were grown in super broth (32 g/L Tryptone, 20 g/L yeast extract, 5 g/L NaCl, pH 7.5) with 100 µg/ml of ampicillin with shaking at 225 rpm at 37°C in 2-L cultures. When  $A_{600}$  reached 0.8-1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and cultures were incubated an additional 2 hr. EDTA and 1,10-o-phenanthroline were added to 5 mM and 0.1 mM respectively, and the bacteria were harvested by centrifugation at 4000 x g for 15 min at 4°C. For extraction of the periplasmic contents, cells were suspended in 75 ml of 20% sucrose containing 30 mM Tris and 1 mM EDTA, incubated at 0° for 10 min, and centrifuged at 8000 x g for 15 min at 4°C. Cells were resuspended gently in 50 ml of cold distilled water, kept on ice for 10 min, and the spheroplasts were pelleted. The supernatant was concentrated with

Centriprep-100 units (Amicon) and loaded on a Sephadryl S-200 column (40 x 2 cm) and 1 ml fractions were collected.

Fractions having full length fusion protein as determined by immunoblots were pooled and concentrated as above. Protein was then purified on an anion exchange column (MonoQ HR5/5, Pharmacia-LKB) using a NaCl gradient. The fusion proteins eluted at 280-300 mM NaCl. The proteins were concentrated again on Centriprep-100 (Amicon Division) and the MonoQ chromatography was repeated. Protein concentrations were determined by the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce), using bovine serum albumin as the standard. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Gels were either stained with Coomassie Brilliant Blue or the proteins were electroblotted to nitrocellulose paper which was probed with polyclonal rabbit antisera to LF or PE (List Biological Laboratories, Campbell, CA). To determine the percent of full length protein, SDS gels stained with Coomassie Brilliant Blue were scanned with a laser densitometer (Pharmacia-LKB Ultrascan XL).

The proteins migrated during gel electrophoresis with molecular masses of more than 106 kDa, consistent with the expected sizes, and immunoblots confirmed that the products had reactivity with antisera to both LF and PE. The fusion proteins differed in their susceptibility to proteolysis as judged by the appearance of smaller fragments on immunoblots, and this led to varying yields of final product. Thus, from 2-L cultures the yields were FP2, 27 µg; FP4, 87 µg; FP23, 18 µg; and FP33, 143 µg.

30 Cell Culture Techniques and Protein Synthesis Inhibition Assay

CHO cells were maintained as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.3), 2 mM glutamine, penicillin/streptomycin, and non-essential amino acids (GIBCO/BRL). Cells were plated in 24- or 48-well dishes one day before the experiment. After overnight incubation, the medium was replaced with fresh medium containing 1 µg/ml

of PA unless otherwise indicated. Fusion proteins were added to 0.1-1000 ng/ml. All data points were done in duplicate. Cells were further incubated for 20 hr at 37°C in 5% CO<sub>2</sub> atmosphere. The medium was then aspirated and cells were  
5 incubated for 2 hr at 37°C with leucine-free medium containing 1  $\mu$ Ci/ml [<sup>3</sup>H]leucine. Cells were washed twice with medium, cold 10% trichloroacetic acid was added for 30 min, the cells were washed twice with 5% trichloroacetic acid and dissolved in 0.150 ml 0.1 M NaOH. Samples were counted in Pharmacia-LKB  
10 1410 liquid scintillation counter. In experiments to determine if the toxin is internalized through acidified endosomes, 1  $\mu$ M monensin (Sigma) was added 90 min prior to toxin and was present during all subsequent steps. To verify that the fusion proteins were internalized through the PA  
15 receptor, competition with native LF was carried out. PA (0.1  $\mu$ g/ml) and LF (0.1-10,000 ng/ml) were added to the CHO cells to block the PA receptor and the fusion proteins were added thereafter at concentrations of 100 ng/ml for FP4 and FP23 and 5 ng/ml for FP33. Protein synthesis inhibition was measured  
20 after 20 hr as described above.

#### Cytotoxic Activity of the Fusion Proteins

All four fusion proteins made and purified were toxic to CHO cells. The concentration causing 50% lysis of cultured cells (EC<sub>50</sub>) values of the proteins were 350, 8, 10, and 0.2 ng/ml for FP2, FP4, FP23 and FP33 respectively (Table 1).  
25 These assays were done with PA present at 1 ug/ml, exceeding the K<sub>m</sub> of 0.1 ug/ml (100 pM). The fusion proteins had no toxicity even at 1  $\mu$ g/ml when PA was omitted, proving that internalization of the fusion proteins was occurring through  
30 the action of PA and the PA receptor. Native LF has previously been shown to have no short-term toxic effects on CHO cells when added with PA, and therefore was not included in these assays. The fusion protein having only domain III and an altered carboxyl-terminus (FP33) was most active,  
35 whereas the one having the intact domains II and III and the native REDLK terminus (FP2) was least active. The other two fusion proteins (FP4 and FP23) had intermediate potencies.

Among proteins having ADP-ribosylation activity, potencies equalling or exceeding 1 pM have previously been found only for native diphtheria and *Pseudomonas* toxins acting on selected cells (Middlebrook, J. L. and Dorlan, R.B. Can. J. Microbiol. 23:183-189, 1977) and for fusion proteins of PE and diphtheria toxin when tested on cells containing > 100,000 receptors for the ligand-recognition domain of the fusion (EGF, transferrin, etc.) (Pastan, I. and FitzGerald, D. Science 254:1173-1177, 1991; Middlebrook, et al. 1977). For CHO cells, the potency of FP33 ( $EC_{50} = 2$  pM) is higher than that of PE itself ( $EC_{50} = 420$  pM), even though CHO cells probably have similar numbers of receptors for both PA and PE (approx. 5,000-20,000). If the intracellular trafficking of native PE delivers less than 5% of the molecules to the cytosol, then the 200-fold greater potency of FP33 suggests that the PA/LF system has an inherently high efficiency of delivery to the cytosol.

A comparison of the potencies of the four fusion proteins shows that inclusion of domain II decreases potency. Thus, the fusion with the lowest potency, FP2, was the one containing intact domains II, Ib, and III. In designing the fusion proteins, all or part of PE domain II and Ib was included in several of the constructs because it could not be assumed that the translocation functions possessed by PA and LF would be able to correctly traffic PE domain III to the cytosol. The combination of domains II, Ib, and III, termed PE40, has been used in a large number of toxic hybrid proteins, by fusion to growth factors, monoclonal antibodies, and other proteins (Pastan et al. 1991; Oeltmann, T. N. and Frankel, A. E. Faseb J. 5:2334-2337, 1991), and some of these fusions have shown substantial potency. Domain II was found to be essential in these hybrid proteins to provide a translocation function not present in the receptor-binding domain to which it was fused. The potency of many of these PE40 fusion proteins appears to require that they be trafficked through the Golgi and ER and proteolytically activated in the same manner as native PE, so as to achieve delivery of domain III to the cytosol. The fact that

inclusion of the entire domain II in the LF fusion protein FP2 instead decreased activity suggests that internalization of the LF fusions occurs through a different route, one that does not easily accommodate all the sequences in domain II.

5 Evidence that structures within PE residues 251-278 inhibit translocation of the LF fusions comes from the 35-fold lower potency of FP2 compared to FP23. One structure that might inhibit translocation of the fusions is the disulfide loop formed by Cys265 and Cys287. In native PE, this  
10 disulfide loop appears to be required for maximum activity. Thus, native PE and TGF- $\alpha$ -PE40 fusions become 10- to 100-fold less toxic if one or both these cysteines are changed to serine. The disulfide loop probably acts to constrain the polypeptide so that Arg276 and Arg279 are susceptible to the  
15 intracellular protease involved in the cleavage that precedes translocation. In contrast, the disulfide loop decreases the potency of the LF fusions, perhaps by preventing the unfolding needed for passage through a protein channel, thereby acting in this situation as a "stop transfer" sequence. FP23, which  
20 lacks Cys265, would not contain the domain II disulfide, and therefore would not be subject to this effect. LF, like PA and EF, contains no cysteines, and would not be prevented by disulfide loops from the complete unfolding needed to pass through a protein channel. The suggestion that disulfide  
25 loops act as stop-transfer signals would predict that the disulfide Cys372-Cys379 in PE domain Ib, which is retained in all four LF fusions would also decrease potency. It should be noted that neither the fusions made here nor the PE40 fusions have been analyzed chemically to determine if the disulfides  
30 in domains II and III are actually formed. If the disulfides do form correctly, it would be predicted that the potencies of all of the fusion proteins, and especially that of FP2, would be increased by treatment with reducing agents. These analyses have not yet been performed. This analysis also  
35 suggests that future LF fusions might be made more potent by omission of domain Ib.

The other structural feature of PE known to affect intracellular trafficking is the carboxyl terminal sequence,

REDLK, that specifies retention in the ER (Chaudhary et al. 1990; Muro et al. 1987). To determine if the trafficking of the LF fusion proteins was similar to that of PE, two of the fusion proteins were designed so as to differ only in the terminal sequence. Replacement of the native sequence by LDER, one that does not function as an ER retention signal, produced the most toxic of the four fusion proteins, FP33. FP4, identical except that it retained a functional REDLK sequence, was 30-fold less potent. These data suggest that sequestration of the REDLK-ended fusions decreased their access to cytosolic EF-2. The implication is that PE may require the REDLK terminus to be delivered to the ER for an obligatory processing step, but then be limited in its final toxic potential by sequestration from its cytosolic target. Finally, this comparison strongly argues that internalization of the LF fusions does not follow the same path as PE.

In designing the fusion proteins described here it was hoped that they would have cytotoxic activity against cells that are unaffected by anthrax lethal toxin, and this was successfully realized as shown by the data obtained with CHO cells. However, prior knowledge about LF did not provide a basis for predicting whether the constructs would retain toxicity toward mouse macrophages, the only cells known to be rapidly killed by anthrax lethal toxin. Macrophages are lysed by lethal toxin in 90-120 minutes, long before any inhibition of protein synthesis resulting from ADP-ribosylation of EF-2 leads to decreases in membrane integrity or viability. This kinetic difference made it possible to test directly for LF action. As discussed above, the fusion proteins purified to remove the ~ 89-kDa LF species formed by proteolysis were not toxic to J774A.1 macrophages. This shows that attachment of a bulky group to the carboxyl terminus of LF eliminates its normal toxic activity. In the absence of any assay for the putative catalytic activity of LF, it is not possible to determine the cause of the loss of LF activity. The inability of the fusions to lyse J774A.1 cells also argues against proteolytic degradation of the fusions either in the medium during incubation with cells or after internalization.

An important result of the invention described here is the demonstration that the anthrax toxin proteins constitute an efficient mechanism for protein internalization into animal cells. The high potency of the present fusion proteins argues 5 that this system is inherently efficient, as well as being amenable to improvement. The high efficiency results in part from the apparent direct translocation from the endosome, without a requirement for trafficking through other intracellular compartments. In addition to its efficiency, 10 the system appears able to tolerate heterologous polypeptides.

Macrophage Lysis Assay of Fusion Proteins

Fusion proteins were assayed for LF functional activity on J774A.1 macrophage cell line in the presence of 1 µg/ml PA. One day prior to use, cells were scraped from 15 flasks and plated in 48-well tissue culture dishes. For cytotoxicity tests, the medium was aspirated and replaced with fresh medium containing 1 µg/ml PA and the LF fusion proteins, and the cells were incubated for 3 hr. All data points were performed in duplicate. To measure the viability of the 20 treated cells, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cells to a final concentration of 0.5 mg/ml, and incubation was continued for an additional 45 min to allow the uptake and oxidation of MTT by viable cells. Medium was aspirated and replaced by 25 200 µl of 0.5% SDS, 40 mM HCl, 90% isopropanol and the plates were vortexed to dissolve the blue pigment. The MTT absorption was read at 570 nm using a UVmax Kinetic Microplate Reader (Molecular Devices Corp.).

The crude periplasmic extracts from which the fusion 30 proteins were purified caused lysis of J774A.1 macrophages when added with PA, indicating the presence of active LF species, probably formed by proteolysis of the fusion proteins. Purification removed this activity, so that none of the final fusion proteins had this activity. This result 35 showed both that the purified proteins were devoid of full size LF or active LF fragments, and that the lytic activity of LF for macrophages is blocked when residues from PE are fused at its carboxyl terminus.

ADP-Ribosylation Assays

For assaying ADP-ribosylation activity, the method of Collier and Kandel (Collier, R. J. and Kandel, J. J. Biol. Chem. 246:1496-1503, 1971) was used with some modification. A wheat germ extract enriched for EF-2 was used in the reaction. Briefly, in a 200- $\mu$ L reaction assay, 20  $\mu$ L of buffer (500 mM Tris, 10 mM EDTA, 50 mM dithiothreitol and 10 mg/ml bovine serum albumin) was mixed with 30  $\mu$ L of EF-2, 130  $\mu$ L of H<sub>2</sub>O or sample, and 20  $\mu$ L of [adenylate-<sup>32</sup>P]NAD (0.4  $\mu$ Ci per assay, ICN Biochemicals) containing 5  $\mu$ M of non-radioactive NAD. Samples were incubated for 20 min at 23°C, the reactions were stopped by adding 1 ml 10% trichloroacetic acid, and the precipitates were collected and washed on GA-6 filters (Gelman Sciences). The filters were washed twice with 70% ethanol, air dried, and the radioactivity measured.

Table 1 shows that all the fusion proteins were equally capable of ADP-ribosylation of EF-2. FP2, which had little cytotoxic activity on CHO cells, still retained full ADP-ribosylation activity. It was also found that treatment with urea and dithiothreitol under conditions that activate the enzymatic activity of native PE, caused no increase in the ADP-ribosylation activity of the fusion proteins, suggesting that the proteins were not folded so as to sterically block the catalytic site.

Effect of Mutant PA on LF-PE Activity

To verify that uptake of the fusion proteins requires PA, the activity of the fusion proteins was measured in the presence of a mutant PA which is apparently defective in internalization. This mutant, PA-S395C, has a serine to cysteine substitution at residue 395 of the mature protein, and retains the ability to bind to receptor, become proteolytically nicked, and bind LF, but is unable to lyse macrophages. When PA-S395C was substituted for native PA in combination with FP33, no inhibition of protein synthesis inhibition was observed. Similar results were obtained when the other three fusion proteins were tested in combination with PA-S395C.

Effect of Monensin on Activity of the Fusion Proteins

To verify that internalization of the fusion proteins was occurring by passage through acidified endosomes in the same manner as native LF, the ability of monensin to protect 5 cells was examined. Addition of monensin to 1  $\mu$ M decreased the potency of FP33 by >100-fold. Protection against the other three fusion proteins exceeded 20-fold.

LF Block of LF-PE Fusion Activity

To further verify that the fusion proteins were 10 internalized through the PA receptor, CHO cells were incubated with PA and different amounts of LF to block the receptor and the fusion proteins were added thereafter. Protein synthesis inhibition assays showed that native LF could competitively block LF-PE fusion proteins in a concentration-dependent 15 manner.

The present data suggest that the receptor-bound 63-kDa proteolytic fragment of PA forms a membrane channel and that regions at or near the amino-termini of LF and EF enter this channel first and thereby cross the endosomal membrane, 20 followed by unfolding and transit of the entire polypeptide to the cytosol. This model differs from that for diphtheria toxin in that the orientation of polypeptide transfer is reversed. Since both EF and LF have large catalytic domains, extending to near their carboxyl termini, it appears probable 25 that the entire polypeptide crosses the membrane. In the LF fusion proteins, the attached PE sequences would be carried along with the LF polypeptide in transiting the channel to the cytosol. Thus, the PA63 protein channel must tolerate diverse amino acid residues and sequences. The data presented is 30 consistent with the mechanism of direct translocation of the LF proteins to the cytosol as suggested herein.

TABLE 1 Cytotoxic and catalytic activity of LF-PE fusion proteins

Prot -ein	Amino acid content			Toxicity (EC <sub>50</sub> ) <sup>b</sup>		ADP- Ribosylation activity (relative)
	LF	Link er	PE	(pM)	ng/ ml	
PE	none	none	1-613	420	23	100 <sup>c</sup>
FP2	776	TR	251-613	2700	350	82
FP4	776	TR	362-613	65	8	105
FP23	776	TR	279-613	70	10	108
FP33	776	TR	362-612 <sup>a</sup>	2	0.2	118

<sup>a</sup>REDLK at carboxyl terminus is changed to LDER.

<sup>b</sup>Data is from this example, except for native PE, which is from data not shown, and is equal to a value previously reported (Moehring, T. J. and Moehring, J. M. Cell 11:447-454, 1977).

<sup>c</sup>ADP-ribosylation was measured using 30 ng of fusion protein in a final volume of 0.200 ml with 5 μM NAD. Results were corrected for the molecular weights of the proteins and normalized to PE.

EXAMPLE 2: Residues 1-254 of Anthrax Toxin Lethal Factor are Sufficient to Cause Cellular Uptake of Fused Polypeptides Reagents and General Procedures

Restriction endonucleases and DNA modifying enzymes  
5 were purchased from GIBCO/BRL, Boehringer Mannheim or New England Biolabs. Low melting point agarose (Sea Plaque) was obtained from FMC Corporation. Oligonucleotides were synthesized on a PCR Mate (Applied Biosystems) and purified with Oligonucleotide Purification Cartridges (Applied  
10 Biosystems). Polymerase chain reactions (PCR) were performed on a thermal cycler (Perkin-Elmer-Cetus) using reagents from U. S. Biochemical Corp. or Perkin-Elmer-Cetus. DNA was amplified as described in Example 1. The DNA was sequenced to confirmed the accuracy of all of the constructs described in  
15 the report. SEQUENASE version 2.0 from U. S. Biochemical Corp. was utilized for the sequencing reactions, and DNA sequencing gels were made with Gel Mix 8 from GIBCO/BRL.  
[<sup>35</sup>S]dATP $\alpha$ S and L-[3,4,5-<sup>3</sup>H]leucine were purchased from Dupont-New England Nuclear. Chinese hamster ovary cells (CHO)  
20 were obtained from Michael Gottesman (NCI, NIH). J774A.1 macrophage cells were obtained from American Type Culture Collection.

Plasmid Construction

Three types of LF protein constructs were made and  
25 analyzed in this report. All the constructs were made by PCR amplification of the desired sequences, using the native LF gene as template. LF proteins deleted at the amino- or carboxyl-terminus were constructed by a single PCR amplification reaction that added restriction sites at the  
30 ends for incorporation of the construct into the expression vector. LF proteins deleted for one or more of the 19-amino acid repeats that comprise residues 308-383 were constructed by ligating the products of two separate PCR reactions that amplified the regions bracketing the deletion. The third  
35 group of constructs were fusions of varying portions of the amino terminus of LF with PE domains Ib and III. Like the internally-deleted LF proteins, these LF-PE fusions were also made by ligation of two separate PCR products. In the latter

two types of constructs, the ligation of the PCR products resulted in addition of a linker, ACGCGT, at the junction points. This introduced two non-native residues, Thr-Arg, between the fused domains. The PCR manipulations also added 5 three non-native amino acids, Met-Val-Pro, as an extension to the native amino terminus on all the constructs described in this report. Addition of this sequence is not likely to alter the activity of the constructs (discussed below). It should be noted that the LF-PE fusions described herein contain this 10 three-residue extension.

For PCR reactions to make deletions of 40 and 78 amino acids from the amino-terminus of LF, two different mutagenic oligonucleotide primers were made which were substantially identical to the LF gene template at the intended new termini, 15 and which added *KpnI* sites at their 5'-ends. Another (non-mutagenic) oligonucleotide primer for introduction of a *BamHI* site at the 3' end of LF was prepared. Similarly, to make deletions at the carboxyl-terminus of LF, two different mutagenic primers were used which truncated LF at residues 729 20 and 693 and introduced a *BamHI* site next to the new 3' ends of the LF gene. A second (non-mutagenic) oligonucleotide primer specific for the amino terminus of LF was made which introduced a *KpnI* site at the 5' end of the gene. All of the primers noted above were used in PCR reactions on a pLF7 25 template (Robertson and Leppla, 1986) to synthesize DNA fragments having *KpnI* and *BamHI* sites at their 5' and 3' ends, respectively. The amplified LF DNAs containing the amino- and carboxyl-terminal deletions were digested with the appropriate restriction enzymes. The expression vector pVEX115f+T 30 (provided by V. K. Chaudhary, NCI, NIH) was cleaved sequentially with *KpnI* and *BamHI* and dephosphorylated. This expression vector contains a T7 promoter, an OmpA signal sequence for protein transport to the periplasm, a multiple cloning site that includes *KpnI* and *BamHI* sites, and a T7 35 transcription terminator. The LF and pVEX115f+T DNA fragments were purified from low melting point agarose, ligated overnight, and transformed into *E. coli* DH5 $\alpha$ . Transformants were screened by restriction digestion to identify the desired

recombinant plasmids. Proteins produced by these constructs are designated according to the amino acid residues retained; for example the LF truncated at residue 693 is designated LF<sup>1-693</sup>. All of the mutant LF proteins described above contain 5 three non-native amino acids, Met-Val-Pro, added to the amino-terminus as a result of the PCR manipulations.

To analyze the role of the repeat region of LF, four different constructs were made: 1., removal of the entire repeat region (LF<sup>1-307</sup>.TR.LF<sup>384-776</sup>), 2., removal of the first 10 repeat (LF<sup>1-307</sup>.TR.LF<sup>327-776</sup>), 3., removal of the last repeat (LF<sup>1-364</sup>.TR.LF<sup>384-776</sup>), and 4., removal of repeats 2-4 (LF<sup>1-326</sup>.TR.LF<sup>384-776</sup>). To construct LF<sup>1-307</sup>.TR.LF<sup>384-776</sup>, four different primers were used in two separate PCR reactions. To amplify LF<sup>1-307</sup>, one oligonucleotide primer was made at the 5'-end of the LF gene which added a *Kpn*I site, and a second 15 primer was constructed at the end of residue 307, introducing an *Mlu*I site. For amplifying LF<sup>384-776</sup>, a third primer was made at residue 384 with an added *Mlu*I site, and the fourth primer was made at the residue 776 which introduced a *Bam*HI site at the end. Two PCR amplifications were done using 20 primers one/two and three/four with pLF7 as template (Robertson and Leppla, 1986). The first amplification reaction was digested with *Kpn*I and *Mlu*I separately, and the second amplification reaction was digested with *Mlu*I and 25 *Bam*HI. The expression vector pVEX115f+T was digested separately with *Kpn*I and *Bam*HI and dephosphorylated. All three fragments were gel purified, ligated overnight at 16°C and transformed into *E. coli* DH5 $\alpha$ . The other three constructs were made by similar strategies. Oligonucleotide primers one 30 and four were the same for all four constructs, whereas primers two and three were changed accordingly. All four constructs contain Met-Val-Pro at the amino terminus of LF and Thr-Arg at the site of the repeat region deletion.

To construct LF-PE fusion proteins, fragments of the 35 LF gene extending from the amino terminus to various lengths were amplified from plasmid pLF7 (Robertson and Leppla, 1986) by PCR using a common oligonucleotide primer that added a *Kpn*I site at the 5' end and mutagenic primers which added *Mlu*I

sites at the intended new 3' ends. The PCR products of the LF gene were digested with *Kpn*I, the DNAs were precipitated, and subsequently digested with *Mlu*I. Domains Ib and III of the PE gene (provided by David FitzGerald, NCI, NIH) were amplified by PCR using primers which added *Mlu*I and *Eco*RI sites at the 5' and 3' ends, respectively. The PCR product of PE was digested with *Mlu*I and *Eco*RI. Similarly, the expression vector pVEX115f+T was digested with *Kpn*I and *Eco*RI. All DNA fragments were purified from low-melting agarose gels, three-fragment ligations were carried out, and the products were transformed into *E. coli* DH5 $\alpha$ . The three constructs described in this example have 254, 198 and 79 amino acids of LF joined with PE domains Ib and III. These fusion proteins are designated LF<sup>1-254</sup>.TR.PE<sup>362-613</sup> (SEQ ID NO:10), LF<sup>1-198</sup>.TR.PE<sup>362-613</sup>, and LF<sup>1-79</sup>.TR.PE<sup>362-613</sup>, respectively. The proteins retain the native carboxyl-terminal sequence of PE, REDLK. It should be noted that these abbreviations do not specify the entire amino acid content of the proteins, because all the constructs also contain Met-Val-Pro, which was added to the amino-terminus of the LF domain by the PCR manipulations.

Expression and Purification of Deleted LF and Fusion Proteins

Recombinant plasmids were transformed into *E. coli* SA2821 (provided by Sankar Adhya, NCI, NIH), a derivative of BL21( $\lambda$ DE3) (Studier and Moffatt, 1986) that lacks the proteases encoded by the *lon*, *OmpT*, and *degP* genes, and has the T7 RNA polymerase gene under control of the *lac* promoter (Strauch et al., 1989). Transformants were grown in super broth with 100  $\mu$ g/ml ampicillin, with shaking at 225 rpm, 37°C, in 2-L cultures. When  $A_{600}$  reached 0.8-1.0, isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 1 mM, and cultures were incubated for an additional 2 h. EDTA and 1,10-o-phenanthroline were added to 5 and 0.1 mM, respectively, and periplasmic protein was extracted as described in Example 1. The supernatant fluids were concentrated by Centriprep-30 units (Amicon) and proteins were purified to near homogeneity by gel filtration (Sephacryl S-200, Pharmacia-LKB) and anion exchange

chromatography (MonoQ, Pharmacia-LKB) as described in Example 1. To determine the percentage of full length protein, SDS gels stained with Coomassie Brilliant Blue were scanned with a laser densitometer (Pharmacia-LKB Ultrascan XL). Western blots were performed as described previously (Singh et al., 1991).

The LF proteins having terminal deletions and the LF-PE fusion proteins were obtained from periplasmic extracts and purified to near homogeneity by gel filtration and anion exchange chromatography. The migration of the proteins was consistent with their expected molecular weights. Immunoblots confirmed that the LF proteins had reactivity with LF antisera, and the LF-PE fusion proteins had reactivity with both LF and PE antisera. Fusion proteins and terminally-deleted LF proteins differed in their susceptibility to proteolysis as judged by the appearance of peptide fragments on the immunoblots, and this was also reflected in the different amounts of purified proteins obtained. Thus, from 2-L cultures the yields of purified proteins were LF<sup>41-776</sup>, 39 µg; LF<sup>79-776</sup>, 32 µg; LF<sup>1-729</sup>, 50 µg; LF<sup>1-693</sup>, 46 µg; LF<sup>1-254</sup>.TR.PE<sup>362-613</sup>, 184 µg; LF<sup>1-198</sup>.TR.PE<sup>362-613</sup>, 80 µg; LF<sup>1-79</sup>.TR.PE<sup>362-613</sup>, 127 µg.

LF proteins deleted in the repeat region were found to be unstable and full size product could not be purified. Therefore, the activities of these proteins were determined by assay of crude periplasmic extracts, and immunoblots were used to estimate the amount of the full size proteins present.

Cytotoxicity on Macrophages of LF Proteins Having Terminal and Internal Deletions

Deleted LF proteins were assayed for LF functional activity on the J774A.1 macrophage cell line in the presence of native PA as described in Example 1. Briefly, cells were plated in 24- or 48-well dishes in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and allowed to grow for 18 h. PA (1 µg/ml) and the mutant LF proteins were added and cells were incubated for 3 h. To measure the viability of the treated cells, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cells

to a final concentration of 0.5 mg/ml. After incubating for 45 min, the medium was aspirated and cells were dissolved in 90% isopropanol, 0.5% SDS, 40 mM HCl, and read at 540 nm using a UVmax Kinetic Microplate Reader (Molecular Devices Corp.).

5 To determine the extent of essential sequences at the amino terminus of LF, the toxicities of the two LF proteins deleted at the amino-terminus were measured in combination with PA in the macrophage lysis assay. Purified LF<sup>41-776</sup> and LF<sup>79-776</sup> were unable to lyse J774A.1 macrophage cells. This  
10 indicates that some portion of the sequence preceding residue 41 is needed to maintain an active LF protein.

15 To examine the role of the carboxyl terminus of LF, two proteins truncated in this region were prepared and analyzed. The proteins LF<sup>1-693</sup> and LF<sup>1-729</sup> were assayed on J774A.1 cells and found to be inactive. This is presumed to be due to inactivation of the putative catalytic domain.

To begin study of the role of the repeat region of LF, four constructs were made having deletions in this region. The proteins expressed from these mutants were unstable. Of 20 the four deleted proteins, only LF<sup>1-307</sup>.TR.LF<sup>327-776</sup> had immunoreactive material at the position expected of intact fusion protein. The amount of intact LF<sup>1-307</sup>.TR.LF<sup>327-776</sup> was similar to that of native LF expressed in the same vector. When these unpurified periplasmic extracts were tested in 25 J774A.1 macrophages, only the native LF control was toxic. LF<sup>1-307</sup>.TR.LF<sup>327-776</sup> did not lyse macrophages even when present at 50-fold higher concentration than that of crude periplasmic protein of LF. Conclusions cannot be drawn about the toxicities of the other three constructs because full size 30 fusion proteins were not present in the periplasmic extracts.  
Cell Culture Techniques and Protein Synthesis Inhibition Assay of Fusion Proteins

CHO cells were maintained as monolayers in  $\alpha$ -modified minimum essential medium ( $\alpha$ -MEM) supplemented with 5% fetal 35 bovine serum, 10 mM HEPES (pH 7.3), and penicillin/streptomycin. Protein synthesis assays were carried out in 24- or 48-well dishes as described in Example 1. CHO cells were incubated with PA (0.1 ug/ml) and varying

concentrations of LF, which is expected to block the receptor. Fusion proteins were added at fixed concentrations, as follows: FP4, 100 ng/ml, FP23, 100 ng/ml, and FP33, 5 ng/ml. Cells were incubated for 20 hr and protein synthesis inhibition was evaluated by [<sup>3</sup>H]leucine incorporation.

5 Cytotoxicity of the LF-PE Fusion Proteins on CHO Cells

The use of fusion proteins provides a more defined method for measuring the translocation of LF, as demonstrated in Example 1 showing that fusions of LF with domains Ib and III of PE are highly toxic. Translocation of these fusions is conveniently measured because domain III blocks protein synthesis by ADP-ribosylation of elongation factor 2. The new fusions containing varying portions of LF fused to PE domains Ib and III were designed to identify the minimum LF sequence able to promote translocation. The EC<sub>50</sub> of LF<sup>1-254</sup>.TR.PE<sup>362-613</sup> (SEQ ID NO: 10) was 1.7 ng/ml, whereas LF<sup>1-198</sup>.TR.PE<sup>362-613</sup> and LF<sup>1-79</sup>.TR.PE<sup>362-613</sup> did not kill 50% of the cells even at a 1200-fold higher concentration. Other constructs were also made and analyzed, containing larger portions of LF fused to PE domains Ib and III, and found those to be equal in potency to LF<sup>1-254</sup>.TR.PE<sup>362-613</sup>. These results show that residues 1-254 contain all the sequences essential for binding to PA63. The fusion proteins had no toxicity in the absence of PA, proving that their internalization absolutely requires interaction with PA.

20 Binding of Fusion Proteins and Deleted LF Proteins to PA

25 Binding of LF proteins to cell bound PA was determined by competition with radiolabeled <sup>125</sup>I-LF. Native LF was radiolabeled ( $3.1 \times 10^6$  cpm/ $\mu$ g protein) using the Bolton-Hunter reagent. Binding studies employed the L6 rat myoblast cell line, which has approximately twice as many receptors as the J774A.1 macrophage line (Singh et al., 1989). For convenience, cells were chemically fixed by a gentle procedure that preserves the binding activity of the receptor as well as the ability of the cell-surface protease to cleave PA to produce receptor-bound PA63. Assays were carried out in 24-well dishes using cells plated in DMEM with 10% fetal bovine serum one day before the experiment. Cell monolayers

were washed twice with Hanks' balanced salt solution (HBSS) containing 25 mM HEPES and were chemically fixed for 30 min at 23° in 10 mM N-hydroxysuccinimide and 30 mM 1-ethyl-3-[3-dimethyl[aminopropyl] carbodiimide, in buffer containing 5 10 mM HEPES, 140 mM NaCl, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>. Monolayers were washed with HBSS containing 25 mM HEPES and the fixative was inactivated by incubating 30 min at 23° in 10 DMEM (without serum) containing 25 mM HEPES. Native PA was added at 1 µg/ml in minimum essential medium containing Hanks' salts, 25 mM HEPES, 1% bovine serum albumin, and a total of 15 4.5 mM NaHCO<sub>3</sub>. Cells were incubated overnight at room temperature to allow binding and cleavage of PA. Cells were washed twice in HBSS and mutant LF proteins (0-5000 ng/ml) along with 50 ng/ml <sup>125</sup>I-LF was added to each well. Cells 20 were further incubated for 5 h, washed three times in HBSS, dissolved in 0.5 ml 1 N NaOH, and counted in a gamma counter (Beckman Gamma 9000).

Using this assay, the LF mutant proteins having amino-terminal deletions were found incapable of binding to PA, thereby explaining their lack of toxicity. Carboxyl-terminal deleted LF proteins did bind to PA in a dose dependent manner, although they had slightly lower affinity than LF. The proteins deleted in the repeat region could not be tested for competitive binding because their instability prevented 25 purification of intact protein.

The EC<sub>50</sub> for LF<sup>1-254</sup>.TR.PE<sup>362-613</sup> binding was found to be 220 ng/ml, which is similar to that of LF, 300 ng/ml. Therefore the binding data correlate well with the toxicity of 30 this construct. In contrast, neither LF<sup>1-198</sup>.TR.PE<sup>362-613</sup> nor LF<sup>1-79</sup>.TR.PE<sup>362-613</sup> bound to PA63 on cells, thereby explaining their lack of toxicity.

EXAMPLE 3: Construction of Genes Encoding PA Fusion Proteins

The genes encoding PA (or PA truncated at the carboxyl terminus to abrogate binding to the PA receptor) and an alternative targeting moiety (a single-chain antibody, growth factor, or other cell type-specific domain) are spliced using conventional molecular biological techniques. The PA gene is 35

readily available, and the genes encoding alternative targeting domains are derived as described below.

Single-chain antibodies (sFv)

See Example 4, below.

5    Growth factors and other targeting proteins

The nucleotide sequences of genes encoding a number of growth factors and other proteins that are targeted to specific cell types or classes are reported in freely accessible databases (e.g., GenBank), and in many cases the 10 genes are available. In circumstances where this is not the case, genes can be cloned from genomic or cDNA libraries, using probes based on the known nucleotide sequence of the gene that codes for the growth factor, or derived from a partial amino acid sequence of the protein (see, e.g. 15 Sambrook, supra.). Alternatively, genes encoding the growth factor or other targeting moiety can be produced *de novo* from chemically synthesized overlapping oligonucleotides, using the preferred codon usage of the expression host. For example, 20 the gene for human epidermal growth factor urogastrone was synthesized from the known amino acid sequence of human urogastrone using yeast preferred codons. The cloned DNA, under control of the yeast GAPDH promoter and yeast ADH-1 terminator, expresses a product having the same properties as natural human urogastrone. The product of this synthesized 25 gene is nearly identical to that of the natural urogastrone, the only difference being that the product of the synthetic gene has a tryptophan at amino acid 13, while the other has a tyrosine (Urdea et al. Proc. Natl. Acad. Sci. USA 80:7461-7465, 1983).

30    Expression of PA Fusion proteins

Once constructed, genes encoding PA-fusion proteins are expressed in *Bacillus anthracis*, and recombinant proteins are purified by one of the following methods: (i) size-based chromatographic separation; (ii) affinity chromatography. In 35 the case of PA-sFv fusions, immobilized metal chelate affinity chromatography may be the purification method of choice, because addition of a string of six histidine residues at the carboxyl terminus of the sFv will have no detrimental effect

on binding to antigen. Additional methods of expression of PA-fusion proteins utilize an *in vitro* rabbit reticulocyte lysate-based coupled transcription/translation system, which has been demonstrated to accurately refold chimeric proteins consisting of an sFv fused to diphtheria toxin, or *Pseudomonas* exotoxin A as demonstrated in Example 4.

Functional testing of PA Fusion proteins

After expression and purification, functionality of PA-fusion proteins are tested by determining their ability to act in concert with an LF-PE fusion protein to inhibit protein synthesis in an appropriate cell line. Using a PA-anti human transferrin receptor sFv fusion as a model, the following properties are examined: (i) Cell type-specificity (protein synthesis should be inhibited in cell lines which express the human transferrin receptor, but not in those which do not); (ii) Independence of toxicity from PA receptor binding (excess free PA should have no effect on toxicity of the PA-sFv/LF-PE complex); (iii) Competitive inhibition by excess free antibody (toxicity should be abrogated in the presence of excess sFv, or the monoclonal antibody from which it was derived). For example such tests are described in Examples 4 and 5. These studies and other studies are used to confirm that PA has been successfully re-routed to an alternative receptor to permit the use of the present anthrax toxin-based cell type-specific cytotoxic agents for the treatment of disease.

EXAMPLE 4: Generating Fusion Proteins with Single-chain Antibodies Reagents

Methionine-free rabbit reticulocyte lysate-based coupled transcription/translation reagents, recombinant ribonuclease inhibitor (rRNasin), and cartridges for the purification of plasmid DNA were purchased from Promega (Madison, WI). Tissue culture supplies were from GIBCO (Grand Island, NY) and Biofluids (Rockville, MD). OKT9 monoclonal antibody was purchased from Ortho Diagnostic Systems (Raritan, NJ). PCR reagents were obtained from by Perkin-Elmer Cetus Instruments (Norwalk, CT), and restriction and nucleic acid modifying enzymes (including M-MLV reverse transcriptase) were

from GIBCO-BRL (Gaithersburg, MD). A Geneclean kit for the recovery of DNA from agarose gels was supplied by BIO 101 (La Jolla, CA). Hybridoma mRNA was isolated using a Fast Trak mRNA isolation kit (Invitrogen, San Diego, CA). All isotopes were purchased from Du Pont-New England Nuclear (Boston, MA), except [Adenylate-<sup>32</sup>P]NAD, which was supplied by ICN Biomedicals (Costa Mesa, CA). *Pseudomonas exotoxin A* was obtained from List Biologicals (Campbell, CA).

Oligonucleotides were synthesized on a dual column Milligen-Bioscience Cyclone Plus DNA synthesizer (Burlington, MA), and purified using OPC cartridges (Applied Biosystems, Foster City, CA). DNA templates were sequenced using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH), and SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 10-20% gradient gels (Daiichi, Tokyo, Japan). After electrophoresis, gels were fixed in 10% methanol/7% acetic acid, and soaked in autoradiography enhancer (Amplify, Amersham Arlington Heights, IL). After drying, autoradiography was performed overnight using X-OMAT AR2 film (Eastman Kodak, Rochester, NY).

#### Plasmids

The vector pET-11d is available from Novagen, Inc., Madison, WI. Plasmids were maintained and propagated in *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA).

#### Cell Lines

K562, a human erythroleukemia-derived cell line [ATCC CCL 243] known to express high levels of the human transferrin receptor at the cell surface, was cultured in RPMI 1640 medium containing 24 mM NaHCO<sub>3</sub>, 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 µg/ml gentamycin. An African green monkey kidney line, Vero (ATCC CCL 81), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented as indicated above. The OKT9 hybridoma (ATCC CRL 8021), which produces a MoAb (IgG<sub>1</sub>) reactive to the human transferrin receptor, was maintained in Iscove's modified Dulbecco's medium containing 20% fetal calf serum, in addition to the supplements described above. All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

Construction of sFv from Hybridomas

Antibody  $V_L$  and  $V_H$  genes were cloned using a modification of a previously described technique (Larrick et al. *Biotechniques* 7:360, 1989; Orlandi et al. *Proc. Natl. Acad. Sci. USA* 86:3833, 1989; Chaudhary et al., 1990). Briefly, mRNA was isolated from  $1 \times 10^8$  antibody producing hybridoma cells, and approximately 3  $\mu$ g was reverse transcribed with M-MLV reverse transcriptase, using random hexanucleotides as primers. The resulting cDNA was screened with two sets of PCR primer pairs designed to ascertain from which Kabat gene family the heavy and light chains were derived (Kabat et al. *Sequences of proteins of immunological interest*. Fifth Edition. (Bethesda, Maryland: U.S. Public Health Service, 1991)). Having identified the most effective primer pairs, cDNA's encoding  $V_L$  and  $V_H$  were spliced, separated by a region encoding a 15 amino acid peptide linker, using a previously described PCR technique known as gene splicing by overlap extension (SOE) (Johnson & Bird *Methods Enzymol.* 203:88, 1991). The sFv gene was then cloned into pET-11d, in frame and on the 5'-side of the PE40 gene, such that expression of the construct should generate an sFv-PE40 fusion protein approximately 70 kDa in size.

Design of primers for PCR amplification of V region genes

The first and third complementarity determining regions (CDRs) of terminally rearranged immunoglobulin variable region genes are flanked by conserved sequences (the first framework region, FR1 on the 5' side of CDR1, and the fourth framework region, FR4, on the 3' side of CDR3).

Although murine variable region genes have been successfully cloned, regardless of family, with just two pairs of highly degenerate primers (one pair for  $V_L$  and another for  $V_H$ ) (Gussow et al. *Cold Spring Harbor Symp. Quant. Biol.* 54:265, 1989; Orlandi et al., 1989; Chaudhary et al., 1990; Batra et al., 1991), the method may not be effective in cases where the number of mismatches between primers and the target sequence is extensive. With this in mind, using the Kabat database of murine V gene sequences the present invention provides a set of ten FR1-derived primers (six for  $V_L$  and four

for  $V_H$ ), such that any of the database sequences selected at random would have a maximum of three mismatches with the most homologous primer. This set of primers can be used effectively to clone V region genes from a number of MoAb secreting cell lines.

5 Assembly of the OKT9 sFv gene

mRNA isolated from the hybridoma secreting the OKT9 MoAb was converted to cDNA as described previously (Larrick et al., 1989; Orlandi et al., 1989; Chaudhary et al., 1990).

10 Despite the fact that CL-UNI is the partnering oligonucleotide in each case, a product the required size (approximately 400 bp) is not produced by  $V_L$  primers IV/VI, IIa or IIb. This suggests that mismatches between these primers and the target sequence were too extensive to allow efficient amplification.

15 A similar argument can be used to explain the failure of  $V_H$  primers I and III to produce the required product. It is clear that primers  $V_L$ -I/III and  $V_H$ -V are most effective at amplifying the OKT9  $V_L$  and  $V_H$  genes respectively. PCR amplified OKT9  $V_L$  and  $V_H$  genes were spliced together using the

20 SOE technique, as previously described (Johnson & Bird, 1991). A synthetic DNA sequence encoding a 15 amino acid linker, was inserted between the variable regions; this linker has been used very effectively in the production of functional sFv (Huston et al., 1991; Johnson & Bird, 1991), and appears to

25 allow the variable chains to assume the optimum orientation for antigen binding. Following splicing of V region genes by the SOE procedure, the DNA fragment encoding the OKT9 sFv was electrophoresed through a 1.5% agarose gel, purified by the Geneclean technique, digested with the appropriate pair of restriction enzymes, and cloned into the pET-11d expression vector in frame and on the 5' side of the PE40 gene.

30 In vitro expression of sFv-PE40 fusion proteins

Plasmid templates were transcribed and translated using a rabbit reticulocyte lysate-based transcription/35 translation system, according to the instructions of the manufacturer, in 96-well microtiter plate format L-[<sup>35</sup>S]methionine-labeled proteins (for analysis by SDS-PAGE) and unlabeled proteins (for enzymatic analysis and bioassay),

were produced in similar conditions, except that the isotope was replaced with 20  $\mu$ M unlabeled L-methionine in the latter case. Control lysate was produced by adding all reagents except plasmid DNA. After translation, unlabeled samples were 5 dialysed overnight at 4°C against phosphate-buffered saline (PBS), pH 7.4 in Spectra/Por 6 MWCO (molecular weight cutoff) 50,000 tubing (Spectrum, Houston, TX).

Constructs incorporating the aberrant kappa transcript will contain a translation termination codon in the  $V_L$  chain 10 as previously described, and would therefore be expected to generate a translation product approximately 12 kDa in size. On the other hand, constructs which have incorporated the productive  $V_L$  gene contain no such termination codon, and a full-length fusion protein (approximately 70 kDa in size) 15 should be produced.

*In vitro* expression studies were used to determine the size of the protein encoded by the OKT9 sFv-PE40 gene. The constructs tested in this experiment clearly produce a protein of approximately 70 kDa, indicating that the clones do not 20 contain the aberrant  $V_L$  gene, and are devoid of frameshift mutations. Of several OKT9 sFv constructs tested, none apparently incorporated the incorrect VL gene. However, in the case of another sFv generated by this method (1B7 sFv, derived from a MoAb which binds to pertussis toxin), the 25 majority of the clones tested produced a 12 kDa protein, and were found to contain the aberrant transcript on DNA sequencing. It should be noted that the 12kDa fragment is frequently obscured in 10-20% gradient gels by unincorporated  $^{35}$ S-methionine which co-migrates with the dye front.

30 Determination of Protein Concentration

The enzymatic activities of fusion proteins were compared with those of known concentrations of PE in an ADP-ribosyl transferase assay, allowing molarities to be determined (Johnson et al. *J. Biol. Chem.* 263:1295-1399, 35 1988). Samples were adjusted to contain equivalent concentrations of lysate, thus maintaining an identical amount of substrate (elongation factor 2) in all cases.

Protein Synthesis Inhibition Assay for Functional sFv-PE40  
Binding

Binding of the OKT9 sFv to the human transferrin receptor was qualitatively determined by assessing the ability of the OKT9 sFv-PE40 fusion protein to inhibit protein synthesis in the K562 cell line. *Pseudomonas exotoxin A* is a bacterial protein which is capable of inhibiting *de novo* protein synthesis in a variety of eukaryotic cell types. The toxin binds to the cell surface, and ultimately translocates to the cytosol where it enzymatically inactivates elongation factor 2. PE40 is a mutant form of exotoxin A which lacks a binding domain, but is enzymatically active, and capable of translocation. Fusion proteins containing PE40 and an alternative binding domain (for example, an sFv to a cell surface receptor) will inhibit protein synthesis in an appropriate cell line only if the sFv binds to a cell-surface antigen which subsequently internalizes into an acidified endosome (Chaudhary et al., 1989). The TfnR is such an antigen, so a qualitative assessment of binding may be determined by measuring the ability of the OKT9 sFv-PE40 fusion protein to inhibit protein synthesis in a cell line like K562, which expresses the TfnR. Protein synthesis inhibition assays were performed as described previously (Johnson et al., 1988). Briefly, samples were serially diluted in ice cold PBS, 0.2% BSA, and 11 $\mu$ l volumes were added to the appropriate well of a 96-well microtiter plate (containing 10<sup>4</sup> cells/100 $\mu$ l/well in leucine-free RPMI 1640). After carefully mixing the contents of each well, the plate was incubated for the indicated time at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Each well was then pulsed with 20 $\mu$ l of L-[<sup>14</sup>C(U)]leucine (0.1  $\mu$ Ci/20 $\mu$ l), incubated for 1 hour, and harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA). Results are expressed as a percentage of the isotope incorporation in cells treated with appropriate concentrations of control dialyzed lysate.

The results of this assay, clearly indicate that OKT9 sFv-PE40 is capable of inhibiting protein synthesis with an IC<sub>50</sub> (the concentration of a reagent which inhibits protein

synthesis by 50%) of approximately  $2 \times 10^{-9}$  M. The toxicity of the fusion protein, but not of PE, was abrogated in the presence of excess OKT9 MoAb (12 µg/ml), indicating that binding is specific for the TfnR. No toxicity was observed  
5 when K562 was substituted with Vero (an African Green monkey cell line which expresses the simian version of the transferrin receptor), indicating that the OKT9 sFv retains the human receptor-specific antigen binding properties of the parent antibody.

Having demonstrated binding of the OKT9 sFv to TfnR, its nucleotide sequence was determined using dideoxynucleotide chain-terminating methods, confirming extensive homology with the respective regions of immunoglobulins of known sequence.  
10

15 EXAMPLE 5: Characterization of single-chain antibody (sFv)-toxin fusion proteins produced in vitro in rabbit reticulocyte lysate

The present invention provides *in vitro* production of proteins containing a toxin domain (derived from Diphtheria toxin (DT) or PE) fused to a domain encoding a single-chain antibody directed against the human transferrin receptor (TfnR). The expression of this antigen on the cell surface is coordinately regulated with cell growth; TfnR exhibits a limited pattern of expression in normal tissue, but is widely distributed on carcinomas and sarcomas (Gatter, et al. *J. Clin. Pathol.* 36:539-545, 1983), and may therefore be a suitable target for immunotoxin-based therapeutic strategies (Johnson, V. G. and Youle, R. J. "Intracellular Trafficking of Proteins" Cambridge Univ. Press, Cambridge England, Steer and Hover eds., pp. 183-225; Batra et al., 1991; Johnson et al.,  
20 1988).  
25  
30

Proteins consisting of a fusion between an sFv directed against the TfnR and either the carboxyl-terminus 40 kDa of PE, or the DT mutant CRM 107 [S(525)F] were expressed in rabbit reticulocyte lysates, and found to be specifically cytotoxic to K562, a cell line known to express TfnR. In comparison, a chimeric protein consisting of a fusion between a second DT mutant, DTM1 [S(508)F, S(525)F] and the E6 sFv

exhibited significantly lower cytotoxicity. Legal  
restrictions imposed on manipulating toxin genes *in vivo*  
previously prevented expression of potentially interesting  
toxin-containing fusion proteins (*Federal Register*  
5 51(88)(III):16961 and Appendix F:16971); the present invention  
provides a novel procedure for *in vitro* gene construction and  
expression which satisfies the regulatory requirements,  
facilitating the first study of the potential of non-truncated  
DT mutants in fusion protein ITs. The present data also  
10 demonstrates that functional recombinant antibodies can be  
generated *in vitro*.

Reagents

DT and PE were purchased from List Biologicals  
(Campbell, CA). Nuclease treated, methionine-free rabbit  
15 reticulocyte lysate and recombinant ribonuclease inhibitor  
(rRNasin) were obtained from Promega (Madison, WI). Tissue  
culture supplies were from GIBCO (Grand Island, NY) and  
Biofluids (Rockville, MD). Reagents for PCR were provided by  
Perkin-Elmer Cetus (Norwalk, CT). Restriction and nucleic  
20 acid modifying enzymes were from Stratagene (La Jolla, CA), as  
was the MCAP kit used to produce capped mRNA *in vitro*.  
Geneclean and RNaid kits (for the purification of DNA and RNA  
respectively) were supplied by BIO 101 (La Jolla, CA). L-[<sup>35</sup>S]methionine, L-[<sup>14</sup>C(U)]leucine and 5'-(*alpha*-thio)-  
25 [<sup>35</sup>S]dATP were from New England Nuclear (Boston, MA).  
[Adenylate-<sup>32</sup>P]NAD was supplied by ICN Biomedicals (Costa  
Mesa, CA).

Oligonucleotide Synthesis

30 Oligonucleotides were synthesized (0.2μM scale), using  
cyanoethylphosphoramidites supplied by Milligen-Bioscience  
(Burlington, MA) on a dual column Cyclone Plus DNA  
synthesizer. Post-synthesis purification was achieved using  
OPC cartridges (Applied Biosystems, Foster City, CA).

Plasmids

35 pET-11d was the generous gift of Dr. F. William  
Studier, Brookhaven National Laboratory (Upton, NY). pHB21-  
PE40, a derivative of pET-11d containing the gene for PE40,  
was kindly supplied by Dr. David FitzGerald (NIH, Bethesda,

MD). All plasmids were maintained and propagated in *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA).

Cell Lines

Corynebacterium diphtheriae strain C7<sub>s</sub>(β)<sup>tox+</sup> (ATCC 27012) was obtained from the ATCC (Rockville, MD), and the strain producing the binding-deficient DT mutant CRM 103 was the generous gift of Dr. Neil Groman, University of Washington (Seattle, WA). Both strains were propagated in LB broth. K562 (a human erythroleukemia-derived cell line, ATCC CCL 243) was cultured in RPMI 1640 medium containing 24 mM NaHCO<sub>3</sub>, 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10 µg/ml gentamycin. Vero (an African green monkey kidney line, ATCC CCL 81) was grown in Dulbecco's modified Eagle's medium supplemented as described above. All eukaryotic cells were cultured at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

Splicing Genes using PCR

Genes encoding antibody V<sub>L</sub> and V<sub>H</sub> were spliced, separated by a region encoding a 15 amino acid peptide linker, using a previously described PCR technique known as gene splicing by overlap extension (SOE) (Horton et al. Gene 77:61-68, 1989; Horton et al. Biotechniques 8:528-535, 1990). For studies requiring *in vitro* expression of PCR products, tox gene-derived fragments were linked to those encoding sFv using a similar method, without the use of restriction enzymes.

Construction of Plasmids Encoding Toxin-sFv Fusion Proteins

The gene encoding PE40 was obtained as an insert in pET-11d, and the sFv gene was cloned on the 5' side of this insert as indicated. To clone the gene encoding the DT binding-site mutant DTM1 [S(508)F, S(525)F], genomic DNA was isolated from the *C. diphtheriae* strain which produces CRM 103. DNA was extracted by a modification of the cetyltrimethylammonium bromide extraction procedure (Wilson, K. "Current Protocols in Molecular Biology" Asubel et al. eds. John Wiley & Sons New York, 2.4.1 - 2.4.5, 1988) and subjected to 20 cycles of PCR amplification. Primers were designed to: (i) amplify the 1605 bp region encoding CRM 103, concomitantly mutating the codon at position 525 from TCT to TTT, and (ii)

incorporate restriction sites appropriate for cloning. The mutations present in CRM 107 and CRM 103 were thus combined on a single gene.

In Vitro Transcription of DNA Templates

5 For transcription, DNA templates required a T7 RNA polymerase promoter immediately upstream of the gene of interest (Oakley, J. L. and Coleman, J. E. *Proc. Acad. Sci. U.S.A.* 74:4266-4270, 1977). Such a promoter was conveniently present in pET-11d (Studier et al. *Enzymol* 185:60-89, 1990).

10 In the case of PCR products, the upstream primer (a 57-mer, T7-DT) was used to introduce all of the elements necessary for *in vitro* transcription/translation. T7-DT includes a consensus T7 RNA polymerase promoter, together with the first seven codons of mature DT (Greenfield et al. *Proc. Natl. Acad. Sci. U.S.A.* 80:6853-6857, 1983) immediately preceded by an ATG translation initiation codon in the optimum Kozak context (Kozak, M. *J. Biol. Chem.* 266:19867-19870, 1991).

15  $m^7G(5')ppp(5')G$ -capped RNA was produced by transcription from linearized plasmids or PCR products using an mCAP kit,

20 according to the manufacturer's protocol. Prior to translation, RNA was purified using an RNaid kit, recovered in nuclease free water, and analyzed by formaldehyde gel electrophoresis.

In Vitro Expression of Fusion Proteins

25 L-[<sup>35</sup>S]methionine-labelled proteins (for analysis by SDS-PAGE) were produced from capped RNA in methionine-free, nuclease treated rabbit reticulocyte lysate, according to the supplier's instructions. Unlabeled proteins (for bioassay), were produced in similar conditions, except that the isotope was replaced with 20  $\mu$ M unlabeled L-methionine. Control lysate was produced by adding all reagents except exogenous RNA. After translation, samples were dialysed overnight at 4°C against PBS, pH 7.4 in Spectra/Por 6 MWCO 50,000 tubing (Spectrum, Houston, TX).

35 Prior to transcription, plasmids were linearized at the *BglII* site and treated with proteinase K to destroy ribonucleases that may contaminate the sample. After phenol/chloroform extraction and ethanol precipitation, DNA

was dissolved in nuclease free water to a concentration of approximately 0.2 µg/µl. m<sup>7</sup>G(5')ppp(5')G-capped RNA was synthesized by T7 RNA polymerase using the conditions recommended by the manufacturer, and its integrity was confirmed by formaldehyde gel electrophoresis. Capped RNA was translated in a commercially available rabbit reticulocyte lysate, according to the instructions of the manufacturer. It is clear from the gel that the major band in each case has a molecular weight corresponding to that of the protein of interest, and that relatively large molecules (approximately 120 kDa in the case of DTM1-E6 sFv-PE40) can be synthesized in the lysate using the conditions described.

Immediately following translation, samples were extensively dialyzed overnight at 4°C against PBS, pH 7.4. The dialysis step was found to be essential, because non-dialyzed rabbit reticulocyte lysate resulted in the incorporation of significantly lower amounts of <sup>14</sup>C-leucine upon assay by protein synthesis inhibition in all cell lines tested. After determining the concentration of the newly synthesized protein using a standard assay for measuring ADP-ribosyltransferase activity (Johnson et al., 1988), the cytotoxic activity of samples was immediately determined.

#### ADP-ribosyl Transferase Assay

The enzymatic activity (and therefore molarity) of fusion proteins was determined by comparison with DT or PE standard curves, as described previously (Johnson et al., 1988). Appropriate volumes of control lysate were added to each standard curve sample, in order to control for the presence of significant levels of EF-2 in reticulocyte lysate.

#### Other Methods

SDS-PAGE was performed as previously described (Laemmli, U. K. *Nature* 227:680-685, 1970), using 10-20% gradient gels (Daiichi, Tokyo, Japan). Once electrophoresis was complete, gels were fixed for 15 minutes in 10% methanol, 3% acetic acid, and then soaked for 30 minutes in autoradiography enhancer (Amplify, Amersham Arlington Heights, IL). After drying, autoradiography was performed overnight using X-OMAT AR2 film (Eastman Kodak, Rochester, NY), in the

absence of intensifying screens. Dideoxynucleotide chain-termination sequencing of double-stranded DNA templates was performed using a Sequenase II kit (United States Biochemical Corp., Cleveland, OH), according to the manufacturer's protocol.

5           Cytotoxicity of Toxin-sFv Fusion Proteins Expressed in Reticulocyte Lysates

The cytotoxic activity of fusion proteins was determined by their ability to inhibit protein synthesis in relevant cell lines (e.g., K562). Assays were performed as described previously (Johnson et al., 1988). Briefly, samples were serially diluted in ice cold PBS, 0.2% BSA, and 11 $\mu$ l volumes were added to the appropriate well of a 96-well microtiter plate (containing 10<sup>4</sup> cells/well in leucine-free RPMI 1640). After carefully mixing the contents of each well, the plate was incubated for the indicated time at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Each well was then pulsed with 20 $\mu$ l of L-[<sup>14</sup>C(U)]leucine (0.1  $\mu$ Ci/20 $\mu$ l), incubated for 1 hour, and harvested onto glass fiber filters using a PHD cell harvester (Cambridge Technology, Cambridge, MA). Results were expressed as a percentage of the isotope incorporation in cells treated with appropriate concentrations of control dialyzed lysate.

25           The results of the protein synthesis inhibition assay clearly indicate that PE40-containing fusion proteins synthesized in cell-free reticulocyte lysates are highly cytotoxic to this cell line ( $IC_{50}$  1 x 10<sup>-10</sup> M). In contrast, DTM1-E6 sFv was at least ten-fold less toxic to K562 than the PE40-containing fusion protein, despite the fact that it exhibited ADP-ribosyl transferase activity indistinguishable from that of wt DT synthesized from an equivalent amount of RNA in an identical reticulocyte lysate mix. Since the decreased toxicity of DTM1-E6 sFv is clearly not due to a deficit in enzymatic activity, the binding and/or translocation process is implicated. Possible mechanisms by which the sFv-antigen interaction could be inhibited include: (i) misfolding of the sFv domain or (ii) steric interactions with other regions of the fusion protein preventing close

association of sFv with the TfnR. It is of interest that a tripartite protein, DTM1-E6 sFv-PE40 was significantly cytotoxic to K562 ( $IC_{50}$  around  $1 \times 10^{-10}$  M, similar to that of PE40-E6 sFv), and the toxic effect was clearly mediated via  
5 the TfnR, since this activity was blocked by addition of excess E6 Mab. Although it is possible that the inclusion of the PE40 moiety at the carboxyl end of the tripartite molecule results in a significant conformational change in domains more proximal to the amino terminus, it seems unlikely that the sFv  
10 binding domain of DTM1-E6 is misfolded, or unavailable to interact with the TfnR. Interactions of DTM1-E6 sFv with the cell surface could be measured in a direct binding assay (Greenfield et al. *Science* 238:536-539, 1987), but these studies were not performed in the course of this  
15 investigation. Nevertheless, it appears likely that the lack of toxicity of the DTM1-E6 sFv fusion protein is due to a deficit in its translocation function.

The expression system developed is rapid and easy, and facilitates the manipulation of a number of samples at once.  
20 No complicated protein purification or refolding procedures are required, and the method can be used to express proteins which, due to restrictions imposed on the manipulation of toxin-encoding genes, could not be produced by more conventional methods. The technique is ideal for ascertaining  
25 the suitability of new sFv for IT development; it is theoretically possible to assemble the sFv-encoding gene (and that encoding the IT itself) by splicing of PCR products derived directly from the hybridoma, without the necessity for cloning. This would facilitate the selection of the most  
30 promising candidate molecule, prior to investing considerable effort and expense in large scale protein production and purification. Toxins and toxin-containing fusion proteins are proving to be powerful aids in our understanding of receptor mediated endocytosis and intracellular routing, and are  
35 providing valuable insight into normal cell function (reviewed in ref. 2). The method described simplifies the generation of such molecules, and facilitates their production and use in

laboratories in which the application of more conventional expression methods would be impractical.

Example 6: Cassette Mutagenesis to Produce PAHIV Mutants.

Three pieces of DNA are joined together. Piece A has vector sequences and encodes the "front half" (5' end of the gene) of PA protein, B is short piece of DNA (referred to as a cassette) and encodes a small middle piece of PA protein and piece C which encodes the "back half" (3' end of the gene) of PA.

PA with alternate HIV-1 cleavage sites were created by a cassette mutagenesis procedure. Eight deoxyoligonucleotides were synthesized for construction of cassettes coding for specifically designed amino acid sequences. All four cassettes were generated by annealing two synthetic oligonucleotides (primers).

Primer 1A CG CAA GTA TCA CAA AAT TAT CCG ATC GTG CAA AAC ATA CTG CAG G  
Q V S O N Y P I V O N I L Q

Primer 1B G TTC CTG CAG TAT GTT TTG CAC GAT CGG ATA ATT TTG TGA TAC TTG

Primer 2A CG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG G  
N T A T I M M O R G N F L Q

Primer 2B G TCC CTG CAG AAA ATT ACC ACG TTG CAT CAT GAT AGT GGC AGT GTT

Primer 3A CG ACT GTC TCT TTT AAC TTC CCG CAA ATC ACG CTT TGG CTG CAG G  
T V S F N F P O I T L W L Q

Primer 3B G TCC CTG CAG CCA AAG CGT GAT TTG CGG GAA GTT AAA AGA GAC AGT

Primer 4A CG GGC GGT TCT GCC TTT AAC TTC CCG ATC GTC ATG GGA GGT CTG CAG G  
G G S A F N F P I V M G G L Q

Primer 4B G TCC CTG CAG ACC TCC CAT GAC GAT CGG GAA GTT AAA GGC AGA ACC GCC

The underlined portion of each protein sequence is recognized and cleaved by the HIV-1 protease.

Primer pair 1 encodes a protein sequence which duplicates part of the cleavage site found between the membrane associated protein and the capsid protein.

Primer pair 2 encodes a protein sequence which duplicates part of the cleavage site between the capsid and the nucleocapsid protein.

Primer pair 3 encodes a protein sequence which 5 duplicates part of the cleavage site between the protease and the p6 protein. Like the protease, p6 is a portion of the large protein produced by HIV.

Primer pair 4 encodes a protein sequence which should 10 be cleaved by the protease. It was created by examining several protein sequences which are recognized by the HIV protease and using the common residues from each sequence. Glycine residues were added to each end to make the molecule more flexible.

The mutagenic cassettes were ligated with the 15 *Bam*HI/*Bst*BI fragment from plasmid pYS5 and the *Ppu*MI-*Bam*I-II fragment from plasmid pYS6. Plasmids shown to have correct restriction maps were transformed into the *E. coli* *dam*<sup>-</sup> *dcm*<sup>-</sup> strain GM2163 (available from New England Bio-Labs, Beverly, MA). Unmethylated plasmid DNA was purified from each mutant 20 and used to transform *B. anthracis*. For methods, see Klimpel, et al. *Proc. Natl. Acad. Sci.* 89:10277-10281 (1992). pYS5 and pYS6 construction are described in Singh, et al. *J. Bio. Chem.* 264:19103-19107 (1989).

The nucleotide and amino acid sequence of the mature 25 PA protein after alteration with primer set 2 are shown below. Nucleotides residues 482 to 523 were replaced with cassette 2 resulting in replacement of amino acid residues 162-171 of PA with residues NTATIMMQRGNFLQ, PAHIV#2. The altered DNA sequence and the new amino acid residues are underlined.

Sequence Range: 1 to 2220

60 \*

5 GAA GTTAAA CAG GAG AAC CGG TTATTAAT GAA TCAGAA TCAAGTTCC CAGGGG TTACTA  
CTT CAATTGTC CTC TTG GCC AATAATTAACTT AGTCTT AGTCAGGGTC CCC AATGAT  
Glu Val Lys Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Gln Gly Leu Leu >

120 \*

10 GGA TACTAT TTT AGT GAT TTG AATTTCAAGCA CCCATG GTGGTTACCTCTTCT ACTACA  
CCT ATGATA AAA TCA CTA AAC TTAAAAGTT CGT GGGTAC CACCAA TGGAGAAGA TGATGT  
Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro Met Val Val Thr Ser Thr Thr >

180 \*

15 GGG GATTTA TCT ATT CCT AGT TCTGAGTTAGAA AAT ATT CCATCG GAAAAC CAA TATTTT  
CCC CTAAAT AGA TAA GGA TCA AGACTCAAT CTT TTATAAGGTAGC CTTTG GTT ATAAAAA  
Gly Asp Leu Ser Ile Pro Ser Ser Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe >

240 \*

20 CAA TCTGCT ATT TGG TCAAGGATTATCAAAGTT AAGAAG AGT GAT GAATATACA TTTGCT  
GTT AGACGA TAA ACC AGT CCT AAATAGTT CAA TTCTTC TCACTACCTTATA TGT AAACGA  
Gln Ser Ala Ile Trp Ser Gly Phe Ile Lys Val Lys Ser Asp Glu Tyr Thr Phe Ala >

300 \*

25 ACT TCCGCT GAT AAT CAT GTA ACAATGTGGGTA GATGAC CAAGAAGTGATT AAT AAAGCT  
TGA AGGCGA CTA TTG GTA CAT TGT TAC ACC CAT CTACTG GTT CTT CACTAATTAA TTTCGA  
Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val Ile Asn Lys Ala >

360 \*

35 TCT AATTCT AAC AAA ATC AGA TTAGAAAAAGGA AGATTA TAT CAA AAAAAATT CAATAT  
AGA TTAAGA TTG TTT TAG TCT AAT CTTTT CCT TCTAAT ATAGTT TATTTT TAA GTTATA  
Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg Leu Tyr Gln Ile Lys Ile Gln Tyr >

420 \*

40 CAA CGAGAA AAT CCT ACT GAAAAAGGATTGGAT TTCAAG TTG TAC TGG ACC GAT TCT CAA  
GTT GCT CTT TTA GGA TGA CTT TTT CCTAAC CTA AAG TTC AACATG ACCTGG CTA AGAGTT  
Gln Arg Glu Asn Pro Thr Glu Lys Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln >

480 \*

45 AAT AAAAAA GAA GTG ATT TCT AGT GATAACTTA CAATTG CCAGAA TTAAAAA CAA AAATCT  
TTA TTTTTT CTT CAC TAA AGA TCAC TATTG AAT GTTAAC GGT CTT AATTGTT GTT TTTAGA  
Asn Lys Lys Glu Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser >

540 \*

50 TCGAACACTGCCACTATCATGATGCAACGTGGTAATTCTCTGCAGGGA CCTACG GTTCCA  
AGCTTG TGACGGTGATAGTACTACGTTGCACCA TTAAAAGACGTC CCT GGATGC CAAGGT  
Ser Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln Gly Pro Thr Val Pro >

600 \*

55 GAC CGTGAC AAT GAT GGA ATC CCTGATTCA TTGAGGTA GAAGGA TATAACG GTT GAT GTC  
CTG GCAC TGTTA CTA CCT TAG GGACTAAGTAAT CTCCAT CTT CCTATATGC CAA CTACAG  
Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val >

660 \*

65 AAA AATAAA AGA ACT TTT CTT TCACCATGGATT TCTAAT ATT CATGAAAAG AAA GGATTA  
TTT TTATTT TCT TGA AAAGAA AGTGGTACCTAA AGATTA TAAGTACTTTCTTT CCTAAT  
Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu >

720 \*

5 ACC AAATAT AAA TCA TCT CCT GAAAAATGGAGC ACGGCT TCTGAT CCGTAC AGT GATTTC  
 TGG TTTATA TTT AGT AGAGGA CTTTTACCTCG TGCCGA AGACTAGGCATG TCA CTAAAG  
 Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe >

780 \*

10 GAA AAGGTT ACA GGA CGG ATT GATAAGAAT GTA TCACCA GAGGCAAGACAC CCC CTTGTG  
 CTT TTCCAA TGT CCT GCC TAA CTATTCTTACAT AGTGGT CTCCGT TCTGTG GGG GAACAC  
 Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val >

840 \*

15 GCA GCTTAT CCG ATT GTA CAT GTAGATATGGAG AATATT ATT CTC TCAAAAAAT GAGGAT  
 CGT CGAATA GGC TAA CAT GTA CATCTATAC CTC TTATAA TAAGAGAGTTTT TTA CTCCTA  
 Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp >

20 900 \*  
 CAA TCCACA CAG AAT ACT GAT AGT GAAACG AGA ACAATA AGT AAA AACT TCT ACAAGT  
 GTT AGGTGT GTC TTA TGA CTA TCAC TTT GCT CTC TGTTAT TCATTT TTATGAAGA TGTCA  
 Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser >

25 960 \*  
 AGG ACACAT ACT AGT GAA GTA CATGGAAATGCA GAAGTG CATGCG TCGTTCTTT GATATT  
 TCC TGTGTA TGA TCA GTT CAT GTACCTTACGT CTTCAC GTACGCAGCAAG AAA CTATAA  
 Arg Thr His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile >

30 1020 \*  
 35 GGT GGGAGT GTA TCT GCA GGA TTTAGTAATTTCG AATTCA AGTACGGTCGCAATT GATCAT  
 CCA CCCTCA CAT AGA CGT CCTAAATCATTAAGC TTAAGT TCATGCCAGCGTTAA CTAGTA  
 Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His >

40 1080 \*  
 TCA CTATCT CTA GCA GGG GAA AGAACTTGGGCT GAAAACA ATGGGT TAAAT ACC GCTGAT  
 AGT GATAGA GAT CGT CCC CTT TCTGAACC CGA CTTTGT TACCAAATTAA TGG CGACTA  
 Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu Asn Thr Ala Asp >

45 1140 \*  
 ACA GCAAGA TTA AAT GCC AAT ATTAGATAT GTA AATACT GGGACGGCTCCAATC TACAAC  
 TGT CGTTCT AAT TTA CGG TTA TAATCTATA CAT TTATGA CCCTGC CGAGGT TAG ATGTTG  
 Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn >

50 1200 \*  
 55 GTG TTACCA ACG ACT TCG TTAGTGTAGGAAAAAATCAA ACACTC GCGACAATT AAAGCT  
 CAC AATGGT TGC TGA AGC AAT CACAATCCTTT TTAGTT TGTGAG CGCTGTTAA TTTCGA  
 Val Leu Pro Thr Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala >

60 1260 \*  
 65 AAG GAAAAC CAA TTA AGT CAA ATACTTGCACCT AATAAT TATTAT CCTTCT AAA AAC TGC  
 TTC CTTTG GTT AAT TCA GTT TATGAACGTGGA TTATTA ATAATAGGAAGA TTT TTGAAC  
 Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu >

1320 \*

GCG CCAATC GCATTA AAT GCA CAAGACGAT TTC AGTTCT ACT CCAATTACA ATG AATTAC  
 CGC GGTTAG CGT AAT TTA CGT GTTCTGCTAAAG TCAAGA TGAGGTAAATGTTAC TTAATG  
 Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr >

1440 \*

5 GGG AATATA GCA ACA TAC AAT TTTGAAAAT GGA AGAGTG AGGGTGGATACA GGC TCGAAC  
 CCC TTATAT GCT TGT ATG TTA AAAC TTTACCT TCTCAC TCC CAC CTATGT CCG AGCTTG  
 Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn

1500 \*

10 TGG AGTGAA GTG TTA CCG CAA ATT CAAGAAACA ACT GCA CGT ATC ATTTT AAT GGAAA  
 ACC TCAC TT CAC AAT GGC GTT TAAGTTCTTGT TGACGT GCATAGT AAAAATTAC CCTTT  
 Trp Ser Glu Val Leu Pro Gln Ile Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys

1560 \*

15 GAT TTAAAT CTG GTA GAA AGG CGGATAGCGGCC GTTAAT CCTAGTGATCCA TTG GAAACG  
 CTA AATTAA GAC CAT CTT TCC GCCTATCGCCGC CAATTG GATCACTAGGTAAAT CTTTGC  
 Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr

20 ACT AAACCG GAT ATG ACA TTA AAAGAAGCC CTT AAAATA GCATTG GATTT AAC GAACCG  
 TGA TTTGGC CTA TAC TGT AAT TTTCTTCGGG GAA TTTTAT CGTAAACCTAAATTG CTTGGC  
 Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Glu Phe Asn Glu Pro

25 AAT GGAAAC TTA CAA TAT CAA GGGAAAGAC ATA ACCGAA TTTGATTTAAT TTC GATCAA  
 TTA CCTTTG AAT GTT ATAGTT CCCTTCTGTAT TGGCTTAAACTAAATTAAAG CTAGTT  
 Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln

30 35 CAA ACATCT CAA AAT ATC AAG AAT CAGTTAGCG GAATTA AAC GCA ACT AAC ATA TATACT  
 GTT TGTAGA GTT TTA TAG TTC TTAGTCAAT CGC CTTAAT TTGCGT TGATTG TAT ATATGA  
 Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr

1740 \*

40 GTA TTAGAT AAA ATC AAA TTA AAT GCA AAAATG AAT ATT TTAATAAGAGAT AAA CGTTTT  
 CAT AAT CTA TTT TAG TTT AAT TTACGTTT TAC TTATAAAATTAT TCTCTA TTT GCAAAA  
 Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe

1860 \*

45 CAT TATGAT AGA AAT AAC ATA GCAGTTGGGGCG GATGAG TCAGTAGTTAAG GAG GCTCAT  
 GTA ATACTA TCT TTA TTG TAT CGTCACCCCGC CTACTC AGTCATCAATTCC CTC CGAGTA  
 His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala His

50 55 AGA GAAGTA ATT AAT TCG TCA ACAGAGGGATT A TTGTTA AAT ATT GATAAG GAT ATAAGA  
 TCT CTTCAT TAA TTA AGC AGT TGT CTC CCTAAAT AACAAAT TTATAACTATT CTA TATTCT  
 Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Asn Ile Asp Lys Asp Ile Arg

1920 \*

60 65 AAA ATATTA TCA GGT TAT ATT GTAGAAATT GAA GATACT GAAGGG CTTAAA GAA GTTATA  
 TTT TATAAT AGT CCA ATA TAA CAT CTTAACTT CTATGA CTT CCC GAATT TCT CAATAT  
 Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile

2040 \*

AAT GACAGA TAT GAT ATG TTG AAT ATT TCTAGT TTACGG CAAGAT GGGAAAA ACA TTTATA  
 TTA CTGTCT ATA CTA TAC AAC TTATAAGA TCA AAT GCC GTT CTACCTTTTGT AAATAT  
 Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile

2100 \*

5 GAT TTTAAA AAA TAT AAT GAT AAATTACCG TTA TATATA AGTAAT CCCAAT TAT AAGGTA  
CTA AAATTT TTT ATA TTA CTA TTAAATGGCAAT ATATAT TCATTAGGGTTAATA TTCCAT  
Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys Val

2160 \*

10 AAT GTATAT GCT GTT ACT AAA GAAAACACT ATT ATTAAT CCTAGTGAGAAT GGG GATACT  
TTA CATATA CGA CAA TGA TTT CTTTGATCAA TAATTAGGATCACTCTTACCC CTATGA  
Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr

2220 \*

15 AGT ACCAAC GGG ATC AAG AAA ATTAAATCTTT TCTAAA AAAGGCTATGAGATA GGATAA  
TCA TGGTTG CCC TAG TTC TTT TAAAATTAGAAA AGATTT TTT CCGATACTCTAT CCTATT  
Ser Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly\*\*\*

20 The above procedure was followed for PAHIV#1, 3 and 4.

Example 7: Cleavage of Mutant PAHIV Proteins in vitro.

The mutated proteins were treated with purified HIV-1 protease and evaluated for their degree of cleavage with respect to time. The purified protease was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, Bethesda, MD. Alternatively, the protease can be purified following the method of Louis, et al., *Euro. J. Biochem.*, 199:361 (1991).

30 Extended incubation (12 hours) of PA or the mutated PA proteins with the purified HIV-1 protease resulted in the appearance of two additional protein fragments that were not anticipated. These two fragments are approximately 53 kilodaltons and 30 kilodaltons in size. This may represent 35 cleavage of PA and mutant PA proteins at a site recognized by the HIV-1 protease between PA residues Y<sup>259</sup> and P<sup>260</sup>. The residues around this cleavage site, <sup>256</sup>VAAAYPIVHV<sup>264</sup>, have not previously been identified as a potential HIV-1 protease cleavage site.

40 Incubation of RAW 264.7 cells (ATCC No. TIB 71) with lethal factor (LF) and HIV-1 protease-cleaved PAHIV#1 or PAHIV#4 caused cell death, demonstrating that the mutated PA proteins are capable of binding to LF and thus the toxic LF/PE fusion proteins. PAHIV, PAHIV#2 and PAHIV#3 have not yet been 45 tested.

Example 8: Evaluation of cytotoxic agents in cell cultures.

The ability of the PA constructs containing the HIV-1-protease cleavage site to promote killing of HIV-1 infected cells is being evaluated in COS-1 cells (ATCC No. CRL 1650) 5 transfected with the vector HIV-gpt. When COS cells are transfected with this plasmid vector they express all the genes for the production of HIV-1 virus particles except the envelope protein, gp160 (Page, K.A., et al., 1990. *J. Virol.* 64:5270-5276). Without the envelope protein the particles are 10 not infectious. These cells express the HIV-1 proteases and properly cleave the viral protein gp55 to gp24 (Page, K.A., et al., 1990. *J. Virol.* 64:5270-5276). These properties make the transfected cells an excellent model system in which to evaluate the ability of protein constructs of the invention to 15 eliminate HIV-1 infected cells from culture.

The COS-1 cells were transfected with the plasmid vector and the resulting cultures are being selected for stable transfectants. The mutated PA proteins (PAHIV#1, PAHIV#2, PAHIV#3 and PAHIV#4) are added to the culture media 20 of growing HIV-gpt transfected COS-1 cells in the presence of the lethal factor fusion protein FP53 (Arora, N. et al. *J. Biol. Chem.* 267:15542 (1992)). Only cells which properly cleave the mutated PA proteins are able to bind the toxin LF fusion protein. The cultures are evaluated for protein 25 expression (an indirect measure of viability) after 36 hours (Arora, N. and S. H. Leppla. 1992. *J. Biol. Chem.* 268:3334).

Example 9: Treatment of an HIV-1 infected patient.

A human patient who is infected with HIV-1 is selected 30 for treatment. Although infected, this particular patient is asymptomatic. The patient weighs 70 kilograms. A dose of 10 micrograms per kilogram or 700 micrograms of a PAHIV in normal saline is prepared. This dosage is injected into the patient intravenously as a bolus. The dose is repeated weekly for a 35 total of 4 to 6 dosages. The patient is evaluated regularly, such as weekly, in terms of his symptoms, physical exam and laboratory analysis according to the clinician's judgment. Tests of particular interest include the patient's complete

blood count and examination for the presence of HIV infection. The treatment regimen can be repeated with or without alterations at the discretion of the clinician.

Incorporated by reference/paragraph before claims

5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described can be used in the practice or  
10 testing of the present invention, the preferred methods and materials are now described. All publications and patent documents referenced in this application are incorporated herein by reference.

15 It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

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Klimpel, Kurt R.  
Arora, Naveen  
Singh, Yogendra  
Nichols, Peter J.

10

(ii) TITLE OF INVENTION: ANTHRAX TOXIN FUSION PROTEINS AND  
RELATED METHODS

15

(iii) NUMBER OF SEQUENCES: 31

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25

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US  
(B) FILING DATE: June 25, 1993  
(C) CLASSIFICATION:

35

(viii) ATTORNEY/AGENT INFORMATION:

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40

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45

## (2) INFORMATION FOR SEQ ID NO:1:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3291 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

60

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus anthracis*

65

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 580..2907  
(D) OTHER INFORMATION: /product= "Lethal Factor"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	AAATTAGGAT TTCGGTTATG TTTAGTATTT TTTTAAAATA ATAGTATTAA ATAGTGGAAAT	60
5	GCAAATGATA AATGGGCTTT AAACAAAACT AATGAAATAA TCTACAAATG GAATTCTCC	120
	AGTTTAGAT TAAACCATAAC CAAAAAAATC ACACGTCAA GAAAAATGAT AGAATCCCTA	180
10	CACTAATTAA CATAACCAAA TTGGTAGTTA TAGGTAGAAA CTTATTTATT TCTATAATAC	240
	CATGAAAAAA AGTAAATATT CTGTTCCATA CTATTTAGT AAATTATTTA GCAAGTAAAT	300
	TTTGGTGTAT AAACAAAGTT TATCTTAATA TAAAAAATTA CTTTACTTTT ATACAGATTA	360
15	AAATGAAAAA TTTTTATGA CAAGAAATAT TGCCCTTAAT TTATGAGGAA ATAAGTAAAAA	420
	TTTCTACAT ACTTTATTT ATTGTTGAAA TGTTCACTTA TAAAAAAGGA GAGATTAAT	480
20	ATGAATATAA AAAAGAATT TATAAAAGTA ATTAGTATGT CATGTTAGT AACAGCAATT	540
	ACTTGAGTG GTCCCGTCTT TATCCCCCTT GTACAGGGG GCG GGC GGT CAT GGT Ala Gly Gly His Gly	594
	1 5	
25	GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA AAT AAA GAT GAG AAT Asp Val Gly Met His Val Lys Glu Lys Lys Asn Lys Asp Glu Asn	642
	10 15 20	
30	AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG GAA GAG CAT TTA AAG Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln Glu Glu His Leu Lys	690
	25 30 35	
35	GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA AAA GGG GAG GAA GCT Glu Ile Met Lys His Ile Val Lys Ile Glu Val Lys Gly Glu Ala	738
	40 45 50	
40	GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG AAA GTA CCA TCT GAT Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu Lys Val Pro Ser Asp	786
	55 60 65	
45	GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG ATA TAT ATT GTG GAT Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys Ile Tyr Ile Val Asp	834
	70 75 80 85	
50	GTG GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA TTA TCT GAA GAT AAG Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala Leu Ser Glu Asp Lys	882
	90 95 100	
55	AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT TTA TTA CAT GAA CAT Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala Leu Leu His Glu His	930
	105 110 115	
60	TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA CTT GTA ATC CAA TCT Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val Leu Val Ile Gln Ser	978
	120 125 130	
65	TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA CTG AAC GTT TAT TAT Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala Leu Asn Val Tyr Tyr	1026
	135 140 145	
70	GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA AGT AAA ATT AAT CAA Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu Ser Lys Ile Asn Gln	1074
	150 155 160 165	
75	CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC ATT AAA AAT GCA TCT Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr Ile Lys Asn Ala Ser	1122
	170 175 180	
80	GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT CAG CTT AAG GAA CAT	1170

	Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn Gln Leu Lys Glu His 185 190 195	
5	CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA AAT AGC AAT GAG GTA Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln Asn Ser Asn Glu Val 200 205 210	1218
10	CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT ATC GAG CCA CAG CAT Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr Ile Glu Pro Gln His 215 220 225	1266
15	CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT TTT AAT TAC ATG GAT Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala Phe Asn Tyr Met Asp 230 235 240 245	1314
20	AAA TTT AAC GAA CAA GAA ATA AAT CTA TCC TTG GAA GAA CTT AAA GAT Lys Phe Asn Glu Gln Glu Ile Asn Leu Ser Leu Glu Glu Leu Lys Asp 250 255 260	1362
25	CAA CGG ATG CTG TCA AGA TAT GAA AAA TGG GAA AAG ATA AAA CAG CAC Gln Arg Met Leu Ser Arg Tyr Glu Lys Trp Glu Lys Ile Lys Gln His 265 270 275	1410
30	TAT CAA CAC TGG AGC GAT TCT TTA TCT GAA GAA GGA AGA GGA CTT TTA Tyr Gln His Trp Ser Asp Ser Leu Ser Glu Glu Gly Arg Gly Leu Leu 280 285 290	1458
35	AAA AAG CTG CAG ATT CCT ATT GAG CCA AAG AAA GAT GAC ATA ATT CAT Lys Lys Leu Gln Ile Pro Ile Glu Pro Lys Lys Asp Asp Ile Ile His 295 300 305	1506
40	TCT TTA TCT CAA GAA GAA AAA GAG CTT CTA AAA AGA ATA CAA ATT GAT Ser Leu Ser Gln Glu Glu Lys Glu Leu Leu Lys Arg Ile Gln Ile Asp 310 315 320 325	1554
45	AGT AGT GAT TTT TTA TCT ACT GAG GAA AAA GAG TTT TTA AAA AAG CTA Ser Ser Asp Phe Leu Ser Thr Glu Glu Lys Glu Phe Leu Lys Lys Leu 330 335 340	1602
50	CAA ATT GAT ATT CGT GAT TCT TTA TCT GAA GAA GAA AAA GAG CTT TTA Gln Ile Asp Ile Arg Asp Ser Leu Ser Glu Glu Glu Lys Glu Leu Leu 345 350 355	1650
55	AAT AGA ATA CAG GTG GAT AGT AGT AAT CCT TTA TCT GAA AAA GAA AAA Asn Arg Ile Gln Val Asp Ser Ser Asn Pro Leu Ser Glu Lys Glu Lys 360 365 370	1698
60	GAG TTT TTA AAA AAG CTG AAA CTT GAT ATT CAA CCA TAT GAT ATT AAT Glu Phe Leu Lys Lys Leu Lys Leu Asp Ile Gln Pro Tyr Asp Ile Asn 375 380 385	1746
65	CAA AGG TTG CAA GAT ACA GGA GGG TTA ATT GAT AGT CCG TCA ATT AAT Gln Arg Leu Gln Asp Thr Gly Gly Leu Ile Asp Ser Pro Ser Ile Asn 390 395 400 405	1794
70	CTT GAT GTA AGA AAG CAG TAT AAA AGG GAT ATT CAA AAT ATT GAT GCT Leu Asp Val Arg Lys Gln Tyr Lys Arg Asp Ile Gln Asn Ile Asp Ala 410 415 420	1842
75	TTA TTA CAT CAA TCC ATT GGA AGT ACC TTG TAC AAT AAA ATT TAT TTG Leu Leu His Gln Ser Ile Gly Ser Thr Leu Tyr Asn Lys Ile Tyr Leu 425 430 435	1890
80	TAT GAA AAT ATG AAT ATC AAT AAC CTT ACA GCA ACC CTA GGT GCG GAT Tyr Glu Asn Met Asn Ile Asn Leu Thr Ala Thr Leu Gly Ala Asp 440 445 450	1938
85	TTA GTT GAT TCC ACT GAT AAT ACT AAA ATT AAT AGA GGT ATT TTC AAT Leu Val Asp Ser Thr Asp Asn Thr Lys Ile Asn Arg Gly Ile Phe Asn	1986

	455	460	465	
5	GAA TTC AAA AAA AAT TTC AAA TAT AGT ATT TCT AGT AAC TAT ATG ATT Glu Phe Lys Lys Asn Phe Lys Tyr Ser Ile Ser Ser Asn Tyr Met Ile 470 475 480 485			2034
10	GTT GAT ATA AAT GAA AGG CCT GCA TTA GAT AAT GAG CGT TTG AAA TGG Val Asp Ile Asn Glu Arg Pro Ala Leu Asp Asn Glu Arg Leu Lys Trp 490 495 500			2082
15	AGA ATC CAA TTA TCA CCA GAT ACT CGA GCA GGA TAT TTA GAA AAT GGA Arg Ile Gln Leu Ser Pro Asp Thr Arg Ala Gly Tyr Leu Glu Asn Gly 505 510 515			2130
20	AAG CTT ATA TTA CAA AGA AAC ATC GGT CTG GAA ATA AAG GAT GTA CAA Lys Leu Ile Leu Gln Arg Asn Ile Gly Leu Glu Ile Lys Asp Val Gln 520 525 530			2178
25	ATA ATT AAG CAA TCC GAA AAA GAA TAT ATA AGG ATT GAT GCG AAA GTA Ile Ile Lys Gln Ser Glu Lys Glu Tyr Ile Arg Ile Asp Ala Lys Val 535 540 545			2226
30	GTG CCA AAG AGT AAA ATA GAT ACA AAA ATT CAA GAA GCA CAG TTA AAT Val Pro Lys Ser Lys Ile Asp Thr Lys Ile Gln Glu Ala Gln Leu Asn 550 555 560 565			2274
35	ATA AAT CAG GAA TGG AAT AAA GCA TTA GGG TTA CCA AAA TAT ACA AAG Ile Asn Gln Glu Trp Asn Lys Ala Leu Gly Leu Pro Lys Tyr Thr Lys 570 575 580			2322
40	CTT ATT ACA TTC AAC GTG CAT AAT AGA TAT GCA TCC AAT ATT GTA GAA Leu Ile Thr Phe Asn Val His Asn Arg Tyr Ala Ser Asn Ile Val Glu 585 590 595			2370
45	AGT GCT TAT TTA ATA TTG AAT GAA TGG AAA AAT AAT ATT CAA AGT GAT Ser Ala Tyr Leu Ile Leu Asn Glu Trp Lys Asn Asn Ile Gln Ser Asp 600 605 610			2418
50	CTT ATA AAA AAG GTA ACA AAT TAC TTA GTT GAT GGT AAT GGA AGA TTT Leu Ile Lys Lys Val Thr Asn Tyr Leu Val Asp Gly Asn Gly Arg Phe 615 620 625			2466
55	GTT TTT ACC GAT ATT ACT CTC CCT AAT ATA GCT GAA CAA TAT ACA CAT Val Phe Thr Asp Ile Thr Leu Pro Asn Ile Ala Glu Gln Tyr Thr His 630 635 640 645			2514
60	CAA GAT GAG ATA TAT GAG CAA GTT CAT TCA AAA GGG TTA TAT GTT CCA Gln Asp Glu Ile Tyr Glu Gln Val His Ser Lys Gly Leu Tyr Val Pro 650 655 660			2562
65	GAA TCC CGT TCT ATA TTA CTC CAT GGA CCT TCA AAA GGT GTA GAA TTA Glu Ser Arg Ser Ile Leu Leu His Gly Pro Ser Lys Gly Val Glu Leu 665 670 675			2610
70	AGG AAT GAT AGT GAG GGT TTT ATA CAC GAA TTT GGA CAT GCT GTG GAT Arg Asn Asp Ser Glu Gly Phe Ile His Glu Phe Gly His Ala Val Asp 680 685 690			2658
75	GAT TAT GCT GGA TAT CTA TTA GAT AAG AAC CAA TCT GAT TTA GTT ACA Asp Tyr Ala Gly Tyr Leu Leu Asp Lys Asn Gln Ser Asp Leu Val Thr 695 700 705			2706
80	AAT TCT AAA AAA TTC ATT GAT ATT TTT AAG GAA GAA GGG AGT AAT TTA Asn Ser Lys Lys Phe Ile Asp Ile Phe Lys Glu Glu Gly Ser Asn Leu 710 715 720 725			2754
85	ACT TCG TAT GGG AGA ACA AAT GAA GCG GAA TTT TTT GCA GAA GCC TTT Thr Ser Tyr Gly Arg Thr Asn Glu Ala Glu Phe Phe Ala Glu Ala Phe 730 735 740			2802

75

	AGG TTA ATG CAT TCT ACG GAC CAT GCT GAA CGT TTA AAA GTT CAA AAA Arg Leu Met His Ser Thr Asp His Ala Glu Arg Leu Lys Val Gln Lys 745 750 755	2850
5	AAT GCT CCG AAA ACT TTC CAA TTT ATT AAC GAT CAG ATT AAG TTC ATT Asn Ala Pro Lys Thr Phe Gln Phe Ile Asn Asp Gln Ile Lys Phe Ile 760 765 770	2898
10	ATT AAC TCA TAAGTAATGT ATTAAGGAA TTCAAATGGA TTTAATAATA Ile Asn Ser 775	2947
15	ATAATAATAA TAATAATAAC GGGACCAGCC ATTATGAAGC AACTAATTCT AGACTTGATA	3007
20	GTAATTCTTG GGAAGCACCA GATAGTGTAA AAGGTGGCAT TGCCAGAACATG ATATTTTATG TGTTCGTTAG ATATGAAGGC AAAAACATG ATCCTGACCT AGAACTTAAT GATAATGTTA TTAATAATTT AATGCCTTT ATAGGAATAT TAGTAAAAGT GCCGAAAAGA TCCTGTTGCA AAGCTTTAA AGAACATATT ATTCTATCAA GTGGCTGTAT ATTTGTGTA ATTTCAATA AATTTGTAA TTAAGCATAAC GTCAAAAAAC CGAAATCTGA GCTC	3067 3127 3187 3247 3291

25 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 30 (A) LENGTH: 776 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys 1 5 10 15
40	Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln 20 25 30
	Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val 35 40 45
45	Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu 50 55 60
	Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys 65 70 75 80
	Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala 85 90 95
55	Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala 100 105 110
	Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val 115 120 125
60	Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala 130 135 140
	Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu 145 150 155 160
65	Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr 165 170 175

	Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn		
	180	185	190
5	Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln		
	195	200	205
	Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr		
	210	215	220
10	Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala		
	225	230	240
	Phe Asn Tyr Met Asp Phe Asn Glu Gln Glu Ile Asn Leu Ser Leu		
	245	250	255
15	Glu Glu Leu Lys Asp Gln Arg Met Leu Ser Arg Tyr Glu Lys Trp Glu		
	260	265	270
20	Lys Ile Lys Gln His Tyr Gln His Trp Ser Asp Ser Leu Ser Glu Glu		
	275	280	285
	Gly Arg Gly Leu Leu Lys Lys Leu Gln Ile Pro Ile Glu Pro Lys Lys		
	290	295	300
25	Asp Asp Ile Ile His Ser Leu Ser Gln Glu Glu Lys Glu Leu Leu Lys		
	305	310	320
	Arg Ile Gln Ile Asp Ser Ser Asp Phe Leu Ser Thr Glu Glu Lys Glu		
	325	330	335
30	Phe Leu Lys Lys Leu Gln Ile Asp Ile Arg Asp Ser Leu Ser Glu Glu		
	340	345	350
35	Glu Lys Glu Leu Leu Asn Arg Ile Gln Val Asp Ser Ser Asn Pro Leu		
	355	360	365
	Ser Glu Lys Glu Lys Glu Phe Leu Lys Lys Leu Lys Leu Asp Ile Gln		
	370	375	380
40	Pro Tyr Asp Ile Asn Gln Arg Leu Gln Asp Thr Gly Gly Leu Ile Asp		
	385	390	400
	Ser Pro Ser Ile Asn Leu Asp Val Arg Lys Gln Tyr Lys Arg Asp Ile		
	405	410	415
45	Gln Asn Ile Asp Ala Leu Leu His Gln Ser Ile Gly Ser Thr Leu Tyr		
	420	425	430
50	Asn Lys Ile Tyr Leu Tyr Glu Asn Met Asn Ile Asn Asn Leu Thr Ala		
	435	440	445
	Thr Leu Gly Ala Asp Leu Val Asp Ser Thr Asp Asn Thr Lys Ile Asn		
	450	455	460
55	Arg Gly Ile Phe Asn Glu Phe Lys Lys Asn Phe Lys Tyr Ser Ile Ser		
	465	470	480
	Ser Asn Tyr Met Ile Val Asp Ile Asn Glu Arg Pro Ala Leu Asp Asn		
	485	490	495
60	Glu Arg Leu Lys Trp Arg Ile Gln Leu Ser Pro Asp Thr Arg Ala Gly		
	500	505	510
65	Tyr Leu Glu Asn Gly Lys Leu Ile Leu Gln Arg Asn Ile Gly Leu Glu		
	515	520	525
	Ile Lys Asp Val Gln Ile Ile Lys Gln Ser Glu Lys Glu Tyr Ile Arg		
	530	535	540

Ile Asp Ala Lys Val Val Pro Lys Ser Lys Ile Asp Thr Lys Ile Gln  
 545 550 555 560

5 Glu Ala Gln Leu Asn Ile Asn Gln Glu Trp Asn Lys Ala Leu Gly Leu  
 565 570 575

Pro Lys Tyr Thr Lys Leu Ile Thr Phe Asn Val His Asn Arg Tyr Ala  
 580 585 590

10 Ser Asn Ile Val Glu Ser Ala Tyr Leu Ile Leu Asn Glu Trp Lys Asn  
 595 600 605

Asn Ile Gln Ser Asp Leu Ile Lys Lys Val Thr Asn Tyr Leu Val Asp  
 610 615 620

15 Gly Asn Gly Arg Phe Val Phe Thr Asp Ile Thr Leu Pro Asn Ile Ala  
 625 630 635 640

Glu Gln Tyr Thr His Gln Asp Glu Ile Tyr Glu Gln Val His Ser Lys  
 20 645 650 655

Gly Leu Tyr Val Pro Glu Ser Arg Ser Ile Leu Leu His Gly Pro Ser  
 660 665 670

25 Lys Gly Val Glu Leu Arg Asn Asp Ser Glu Gly Phe Ile His Glu Phe  
 675 680 685

Gly His Ala Val Asp Asp Tyr Ala Gly Tyr Leu Leu Asp Lys Asn Gln  
 30 690 695 700

Ser Asp Leu Val Thr Asn Ser Lys Lys Phe Ile Asp Ile Phe Lys Glu  
 705 710 715 720

Glu Gly Ser Asn Leu Thr Ser Tyr Gly Arg Thr Asn Glu Ala Glu Phe  
 35 725 730 735

Phe Ala Glu Ala Phe Arg Leu Met His Ser Thr Asp His Ala Glu Arg  
 740 745 750

40 Leu Lys Val Gln Lys Asn Ala Pro Lys Thr Phe Gln Phe Ile Asn Asp  
 755 760 765

Gln Ile Lys Phe Ile Ile Asn Ser  
 45 770 775

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
 50 (A) LENGTH: 4235 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

60 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Bacillus anthracis*

65 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1891..4095  
 (D) OTHER INFORMATION: /product= "Protective Antigen"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	AAGCTTCTGT CATTCTGAAA TTTCATATAG AACGTAATT TAGACTCTC ATCATTAAAA	60
	ATGAAAATC TTATCTTTT GATTCTATTG TATACTTTA TTAAGGTGTT TAATAGTTAG	120
5	AAAAGACAGT TGATGCTATT ACTCCAGATA AAATATAGCT AACCATAAAAT TTATTAAAGA	180
	AACCTTGTG TTCTAAATAA TGATTTGTG GATTCCGAA TAGACTGG TGAGTTAGCT	240
10	CTAATTTAT AGTGATTAA CTAACAATT ATAAAGCAGC ATAATTCAAATTTTTAATT	300
	GATTTTCCT GAAGCATAGT ATAAAAGAGT CAAGGTCTTC TAGACTTGAC TCTTGGAAATC	360
	ATTAGGAATT ACAATATAT ATAATGCGCT AGACAGAATC AAATTAAATG CAAAAATGAA	420
15	TATTTAGTA AGAGATCCAT ATCATTATGA TAATAACGGT AATATTGTAG GGGTTGATGA	480
	TTCATATTTA AAAAACGCAT ATAAGCAAAT ACTTAATTGG TCAAGCGATG GAGTTCTTT	540
	AAATCTAGAT GAAGATGTAATCAAGCACT ATCTGGATAT ATGCTTCAAA TAAAAAAACC	600
20	TTCAAACAC CTAACAAACA GCCCAGTTAC AATTACATTA GCAGGCAAGG ACAGTGGTGT	660
	TGGAGAATTG TATAGAGTAT TATCAGATGG AGCAGGATTC CTGGATTTCA ATAAGTTGA	720
25	TGAAAATTGG CGATCATTAG TAGATCCTGG TGATGATGTT TATGTGTATG CTGTTACTAA	780
	AGAAGATTT AATGCAGTTA CTCGAGATGA AAATGGTAAT ATAGCGAATA AATTAAAAAA	840
	CACCTTAGTT TTATCGGGTA AAATAAAAGA AATAAACATA AAAACTACAA ATATTAATAT	900
30	ATTTGTAGTT TTTATGTTA TTATATACCT CCTATTTAT ATTATTAGTA GCACAGTTT	960
	TGCAAATCAT GTAATTGTAT ACTTATCTAT GTAGAGGTAT CACAACTTAT GAATAGTGT	1020
35	TTTTATTGAA CGTTGGTTAG CTTGGACAGT TGTATGGATA TGCATACTTT ATAACGTATA	1080
	AAATTCACG CACCACAATA AAACAAATT AACAACAA AAAACACACC TAAGATCATT	1140
40	CAGTTCTTT AATAAGGAGC TGCCCACCAA GCTAACCTA AATAATCTTT GTTTCACATA	1200
	AGGTTTTTCTAAATAC AGTGTAAGTT ATTGTGAATT TAACCAGTAT ATATTAACAA	1260
	TGTTTTATGT TAACAAATTA AATTGTAAAA CCCCTCTAA GCATAGTTAA GAGGGTAGG	1320
45	TTTTAAATT TTTGTGAAA TTAGAAAAAA TAATAAAAAAA ACAACCTAT TTTCTTTCA	1380
	GTTGTTTTG GGTTACAAAA CAAAAAGAAA ACATGTTCA AGGTACAATA ATTATGGTTC	1440
	TTTAGCTTTC TGTAAAACAG CCTTAATAGT TGGATTTATG ACTATTAAAG TTAGTATACA	1500
50	GCATACACAA TCTATTGAAG GATATTTATA ATGCAATTCC CTAAAAATAG TTTGTATAA	1560
	CCAGTTCTTT TATCCGAACG GATACACGTA TTTAGCATA ATTTTAATG TATCTTC	1620
55	AACAGCTTCT GTGTCCTTT CTATTAACAA TATAAAATCT TTTTTATGTT ATATATTAT	1680
	AAAAGTTCTG TTAAAAAGC CAAAAATAAA TAATTATCTC TTTTTATTTA TATTATATG	1740
60	AAACTAAAGT TTATTAATTT CAATATAATA TAAATTTAAT TTTATACAAA AAGGAGAACG	1800
	TATATGAAAA AACGAAAAGT GTTAATACCA TTAATGGCAT TGTCTACGAT ATTAGTTCA	1860
	AGCACAGGTA ATTTAGAGGT GATTCAAGGCA GAA GTT AAA CAG GAG AAC CGG TTA	1914
65	Glu Val Lys Gln Glu Asn Arg Leu 1 5	
	TTA AAT GAA TCA GAA TCA AGT TCC CAG GGG TTA CTA GGA TAC TAT TTT	1962
	Leu Asn Glu Ser Glu Ser Ser Gln Gly Leu Leu Gly Tyr Tyr Phe	

10 15 20

	AGT GAT TTG AAT TTT CAA GCA CCC ATG GTG GTT ACC TCT TCT ACT ACA Ser Asp Leu Asn Phe Gln Ala Pro Met Val Val Thr Ser Ser Thr Thr 25 30 35 40	2010
5	GGG GAT TTA TCT ATT CCT AGT TCT GAG TTA GAA AAT ATT CCA TCG GAA Gly Asp Leu Ser Ile Pro Ser Ser Glu Leu Glu Asn Ile Pro Ser Glu 45 50 55	2058
10	AAC CAA TAT TTT CAA TCT GCT ATT TGG TCA GGA TTT ATC AAA GTT AAG Asn Gln Tyr Phe Gln Ser Ala Ile Trp Ser Gly Phe Ile Lys Val Lys 60 65 70	2106
15	AAG AGT GAT GAA TAT ACA TTT GCT ACT TCC GCT GAT AAT CAT GTA ACA Lys Ser Asp Glu Tyr Thr Phe Ala Thr Ser Ala Asp Asn His Val Thr 75 80 85	2154
20	ATG TGG GTA GAT GAC CAA GAA GTG ATT AAT AAA GCT TCT AAT TCT AAC Met Trp Val Asp Asp Gln Glu Val Ile Asn Lys Ala Ser Asn Ser Asn 90 95 100	2202
25	AAA ATC AGA TTA GAA AAA GGA AGA TTA TAT CAA ATA AAA ATT CAA TAT Lys Ile Arg Leu Glu Lys Gly Arg Leu Tyr Gln Ile Lys Ile Gln Tyr 105 110 115 120	2250
30	CAA CGA GAA AAT CCT ACT GAA AAA GGA TTG GAT TTC AAG TTG TAC TGG Gln Arg Glu Asn Pro Thr Glu Lys Gly Leu Asp Phe Lys Leu Tyr Trp 125 130 135	2298
35	ACC GAT TCT CAA AAT AAA GAA GTG ATT TCT AGT GAT AAC TTA CAA Thr Asp Ser Gln Asn Lys Lys Glu Val Ile Ser Ser Asp Asn Leu Gln 140 145 150	2346
40	TTG CCA GAA TTA AAA CAA AAA TCT TCG AAC TCA AGA AAA AAG CGA AGT Leu Pro Glu Leu Lys Gln Lys Ser Ser Asn Ser Arg Lys Lys Arg Ser 155 160 165	2394
45	ACA AGT GCT GGA CCT ACG GTT CCA GAC CGT GAC AAT GAT GGA ATC CCT Thr Ser Ala Gly Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro 170 175 180	2442
50	GAT TCA TTA GAG GTA GAA GGA TAT ACG GTT GAT GTC AAA AAT AAA AGA Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg 185 190 195 200	2490
55	ACT TTT CTT TCA CCA TGG ATT TCT AAT ATT CAT GAA AAG AAA GGA TTA Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu 205 210 215	2538
60	ACC AAA TAT AAA TCA TCT CCT GAA AAA TGG AGC ACG GCT TCT GAT CCG Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro 220 225 230	2586
65	TAC AGT GAT TTC GAA AAG GTT ACA GGA CGG ATT GAT AAG AAT GTA TCA Tyr Ser Asp Phe Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser 235 240 245	2634
70	CCA GAG GCA AGA CAC CCC CTT GTG GCA GCT TAT CCG ATT GTA CAT GTA Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His Val 250 255 260	2682
75	GAT ATG GAG AAT ATT ATT CTC TCA AAA AAT GAG GAT CAA TCC ACA CAG Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln 265 270 275 280	2730
80	AAT ACT GAT AGT GAA ACG AGA ACA ATA AGT AAA AAT ACT TCT ACA AGT Asn Thr Asp Ser Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser 285 290 295	2778
85	AGG ACA CAT ACT AGT GAA GTA CAT GGA AAT GCA GAA GTG CAT GCG TCG	2826

	Arg Thr His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser					
	300	305	310			
5	TTC TTT GAT ATT GGT GGG AGT GTA TCT GCA GGA TTT AGT AAT TCG AAT Phe Phe Asp Ile Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn	315	320	325	2874	
10	TCA AGT ACG GTC GCA ATT GAT CAT TCA CTA TCT CTA GCA GGG GAA AGA Ser Ser Thr Val Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg	330	335	340	2922	
15	ACT TGG GCT GAA ACA ATG GGT TTA AAT ACC GCT GAT ACA GCA AGA TTA Thr Trp Ala Glu Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu	345	350	355	360	2970
20	AAT GCC AAT ATT AGA TAT GTA AAT ACT GGG ACG GCT CCA ATC TAC AAC Asn Ala Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn	365	370	375	3018	
25	GTG TTA CCA ACG ACT TCG TTA GTG TTA GGA AAA AAT CAA ACA CTC GCG Val Leu Pro Thr Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala	380	385	390	3066	
30	ACA ATT AAA GCT AAG GAA AAC CAA TTA AGT CAA ATA CTT GCA CCT AAT Thr Ile Lys Ala Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn	395	400	405	3114	
35	AAT TAT TAT CCT TCT AAA AAC TTG GCG CCA ATC GCA TTA AAT GCA CAA Asn Tyr Tyr Pro Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln	410	415	420	3162	
40	GAC GAT TTC AGT TCT ACT CCA ATT ACA ATG AAT TAC AAT CAA TTT CTT Asp Asp Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr Asn Gln Phe Leu	425	430	435	440	3210
45	GAG TTA GAA AAA ACG AAA CAA TTA AGA TTA GAT ACG GAT CAA GTA TAT Glu Leu Glu Lys Thr Lys Gln Leu Arg Leu Asp Thr Asp Gln Val Tyr	445	450	455	3258	
50	GGG AAT ATA GCA ACA TAC AAT TTT GAA AAT GGA AGA GTG AGG GTG GAT Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val Asp	460	465	470	3306	
55	ACA GGC TCG AAC TGG AGT GAA GTG TTA CCG CAA ATT CAA GAA ACA ACT Thr Gly Ser Asn Trp Ser Glu Val Leu Pro Gln Ile Gln Glu Thr Thr	475	480	485	3354	
60	GCA CGT ATC ATT TTT AAT GGA AAA GAT TTA AAT CTG GTA GAA AGG CGG Ala Arg Ile Ile Phe Asn Gly Lys Asp Leu Asn Leu Val Glu Arg Arg	490	495	500	3402	
65	ATA GCG GCG GTT AAT CCT AGT GAT CCA TTA GAA ACG ACT AAA CCG GAT Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr Thr Lys Pro Asp	505	510	515	520	3450
70	ATG ACA TTA AAA GAA GCC CTT AAA ATA GCA TTT GGA TTT AAC GAA CCG Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu Pro	525	530	535	3498	
75	AAT GGA AAC TTA CAA TAT CAA GGG AAA GAC ATA ACC GAA TTT GAT TTT Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp Phe	540	545	550	3546	
80	AAT TTC GAT CAA CAA ACA TCT CAA AAT ATC AAG AAT CAG TTA GCG GAA Asn Phe Asp Gln Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala Glu	555	560	565	3594	
85	TTA AAC GCA ACT AAC ATA TAT ACT GTA TTA GAT AAA ATC AAA TTA AAT Leu Asn Ala Thr Asn Ile Tyr Thr Val Leu Asp Lys Ile Lys Leu Asn				3642	

	570	575	580	
	GCA AAA ATG AAT ATT TTA ATA AGA GAT AAA CGT TTT CAT TAT GAT AGA Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe His Tyr Asp Arg			3690
5	585	590	595	600
	AAT AAC ATA GCA GTT GGG GCG GAT GAG TCA GTA GTT AAG GAG GCT CAT Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala His			3738
	605	610		615
10	AGA GAA GTA ATT AAT TCG TCA ACA GAG GGA TTA TTG TTA AAT ATT GAT Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Asn Ile Asp			3786
	620	625		630
15	AAG GAT ATA AGA AAA ATA TTA TCA GGT TAT ATT GTA GAA ATT GAA GAT Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu Asp			3834
	635	640		645
20	ACT GAA GGG CTT AAA GAA GTT ATA AAT GAC AGA TAT GAT ATG TTG AAT Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg Tyr Asp Met Leu Asn			3882
	650	655		660
25	ATT TCT AGT TTA CGG CAA GAT GGA AAA ACA TTT ATA GAT TTT AAA AAA Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile Asp Phe Lys Lys			3930
	665	670		675
	TAT AAT GAT AAA TTA CCG TTA TAT ATA AGT AAT CCC AAT TAT AAG GTA Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys Val			3978
	685	690		695
30	AAT GTA TAT GCT GTT ACT AAA GAA AAC ACT ATT ATT AAT CCT AGT GAG Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser Glu			4026
	700	705		710
35	AAT GGG GAT ACT AGT ACC AAC GGG ATC AAG AAA ATT TTA ATC TTT TCT Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe Ser			4074
	715	720		725
40	AAA AAA GGC TAT GAG ATA GGA TAAGGTAATT CTAGGTGATT TTTAAATTAT Lys Lys Gly Tyr Glu Ile Gly			4125
	730	735		
	CTAAAAAAACA GTAAAATTAA AACATACTCT TTTTGTAAGA AATACAAGGA GAGTATGTT			4185
45	TAAACAGTAA TCTAAATCAT CATAATCCTT TGAGATTGTT TGTAGGATCC			4235

## (2) INFORMATION FOR SEQ ID NO:4:

50	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 735 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
55	(ii) MOLECULE TYPE: protein			
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:			
60	Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Ser Ser 1 5 10 15			
	Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro 20 25 30			
65	Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser 35 40 45			
	Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile 50 55 60			

Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala  
 65 70 75 80

5 Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val  
 85 90 95

Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg  
 100 105 110

10 Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys  
 115 120 125

Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu  
 130 135 140

15 Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser  
 145 150 155 160

20 Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro  
 165 170 175

Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr  
 180 185 190

25 Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser  
 195 200 205

Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu  
 210 215 220

30 Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr  
 225 230 235 240

35 Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val  
 245 250 255

Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser  
 260 265 270

40 Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr  
 275 280 285

Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His  
 290 295 300

45 Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val  
 305 310 315 320

50 Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His  
 325 330 335

Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu  
 340 345 350

55 Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn  
 355 360 365

Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val  
 370 375 380

60 Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln  
 385 390 395 400

65 Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu  
 405 410 415

Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile  
 420 425 430

Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu  
 435 440 445  
 Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe  
 5 450 455 460  
 Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val  
 465 470 475 480  
 Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys  
 10 485 490 495  
 Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp  
 500 505 510  
 15 Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys  
 515 520 525  
 Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly  
 20 530 535 540  
 Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln  
 545 550 555 560  
 Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr  
 25 565 570 575  
 Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg  
 580 585 590  
 30 Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp  
 595 600 605  
 Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr  
 35 610 615 620  
 Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser  
 625 630 635 640  
 Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile  
 40 645 650 655  
 Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly  
 660 665 670  
 45 Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr  
 675 680 685  
 Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu  
 50 690 695 700  
 Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly  
 705 710 715 720  
 Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly  
 55 725 730 735

## (2) INFORMATION FOR SEQ ID NO:5:

60           (i) SEQUENCE CHARACTERISTICS:  
               (A) LENGTH: 1368 base pairs  
               (B) TYPE: nucleic acid  
               (C) STRANDEDNESS: single  
               (D) TOPOLOGY: linear

65           (ii) MOLECULE TYPE: DNA (genomic)

              (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Bacillus anthracis*

5

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1368

(D) OTHER INFORMATION: /product=  
10 "LF(1-254)--TR--PE(401-602)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15	GGC GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys 1 5 10 15	48
20	AAT AAA GAT GAG AAT AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln 20 25 30	96
25	GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val 35 40 45	144
30	AAA GGG GAG GAA GCT GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu 50 55 60	192
35	AAA GTA CCA TCT GAT GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys 65 70 75 80	240
40	ATA TAT ATT GTG GAT GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala 85 90 95	288
45	TTA TCT GAA GAT AAG AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala 100 105 110	336
50	TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val 115 120 125	384
55	CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala 130 135 140	432
	CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu 145 150 155 160	480

	AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr 165 170 175	528
5	ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn 180 185 190	576
10	CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln 195 200 205	624
15	AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr 210 215 220	672
20	ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala 225 230 235 240	720
25	TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT CTA CTC GGC Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Leu Gly 245 250 255	768
30	GAC GGC GGG GAC GTC AGC TTC AGC ACC CGC GGC ACG CAG AAC TGG ACG Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr 260 265 270	816
35	GTG GAG CGG CTG CTC CAG GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr 275 280 285	864
40	GTG TTC GTC GGC TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile 290 295 300	912
45	GTC TTC GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp 305 310 315 320	960
50	CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC TAC GCC Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Ile Ala Tyr Gly Tyr Ala 325 330 335	1008
55	CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC AAC GGT GCC CTG Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu 340 345 350	1056
60	CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG CCG GGC TTC TAC CGC ACC Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr 355 360 365	1104
65	AGC CTG ACC CTG GCC GCG CCG GAG GCG GCG GGC GAG GTC GAA CGG CTG Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu 370 375 380	1152
70	ATC GGC CAT CCG CTG CCG CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu 385 390 395 400	1200
75	GAG GAA GGC GGG CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu 405 410 415	1248
80	CGC ACC GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val 420 425 430	1296
85	GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG GCG ATC	1344

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile  
 435 440 445

5 AGC GCC CTG CCG GAC TAC GCC AGC 1368  
 Ser Ala Leu Pro Asp Tyr Ala Ser  
 450 455

## (2) INFORMATION FOR SEQ ID NO:6:

10 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 456 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

20 Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys  
 1 5 10 15

Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln  
 20 25 30

25 Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val  
 35 40 45

30 Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu  
 50 55 60

Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys  
 65 70 75 80

35 Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala  
 85 90 95

40 Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala  
 100 105 110

Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val  
 115 120 125

45 Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala  
 130 135 140

Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu  
 145 150 155 160

50 Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr  
 165 170 175

Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn  
 180 185 190

55 Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln  
 195 200 205

60 Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr  
 210 215 220

Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala  
 225 230 235 240

65 Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Leu Gly  
 245 250 255

Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp Thr  
 260 265 270

Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly Tyr  
 275 280 285  
 5 Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile  
 290 295 300  
 Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp  
 305 310 315 320  
 10 Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala  
 325 330 335  
 Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu  
 340 345 350  
 15 Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr  
 355 360 365  
 Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu  
 20 370 375 380  
 Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu  
 385 390 395 400  
 25 Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu  
 405 410 415  
 Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val  
 420 425 430  
 30 Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln Ala Ile  
 435 440 445  
 35 Ser Ala Leu Pro Asp Tyr Ala Ser  
 450 455

## (2) INFORMATION FOR SEQ ID NO:7:

40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1425 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

50 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Bacillus anthracis*

55 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1416  
 (D) OTHER INFORMATION: /product=  
 "LF(1-254) --TR--PE(398-613)"

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
 ATG GTA CCA GCG GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG  
 Met Val Pro Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu  
 1 5 10 15

65 AAA GAG AAA AAT AAA GAT GAG AAT AAG AGA AAA GAT GAA GAA CGA AAT  
 Lys Glu Lys Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn  
 20 25 30

AAA ACA CAG GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA

48

96

144

88

	Lys Thr Gln Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys			
	35	40	45	
5	ATA GAA GTA AAA GGG GAG GAA GCT GTT AAA AAA GAG GCA GCA GAA AAG Ile Glu Val Lys Gly Glu Ala Val Lys Lys Glu Ala Ala Glu Lys		192	
	50	55	60	
10	CTA CTT GAG AAA GTA CCA TCT GAT GTT TTA GAG ATG TAT AAA GCA ATT Leu Leu Glu Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile		240	
	65	70	75	80
	GGA GGA AAG ATA TAT ATT GTG GAT GGT GAT ATT ACA AAA CAT ATA TCT Gly Gly Lys Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser		288	
	85	90	95	
15				

	TTA GAA GCA TTA TCT GAA GAT AAG AAA AAA ATA AAA GAC ATT TAT GGG Leu Glu Ala Leu Ser Glu Asp Lys Lys Lys Ile Lys Asp Ile Tyr Gly 100 105 110	336
5	AAA GAT GCT TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT Lys Asp Ala Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr 115 120 125	384
10	GAA CCC GTA CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT Glu Pro Val Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr 130 135 140	432
15	GAA AAG GCA CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG Glu Lys Ala Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg 145 150 155 160	480
	GAT ATT TTA AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA Asp Ile Leu Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val 165 170 175	528
20	TTA AAT ACC ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA Leu Asn Thr Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu 180 185 190	576
25	TTT ACT AAT CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC Phe Thr Asn Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe 195 200 205	624
30	TTG GAA CAA AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT Leu Glu Gln Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe 210 215 220	672
35	GCA TAT TAT ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA Ala Tyr Tyr Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala 225 230 235 240	720
	CCG GAA GCT TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT Pro Glu Ala Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn. 245 250 255	768
40	CTA ACG CGT GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC Leu Thr Arg Ala Glu Phe Leu Gly Asp Gly Asp Val Ser Phe Ser 260 265 270	816
45	ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG GCG CAC Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His 275 280 285	864
50	CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC TAC CAC GGC ACC Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr 290 295 300	912
55	TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC GGC GGG GTG CGC GCG CGC Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg 305 310 315 320	960
	AGC CAG GAC CTC GAC GCG ATC TGG CGC GGT TTC TAT ATC GCC GGC GAT Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp 325 330 335	1008
60	CCG GCG CTG GCC TAC GGC TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg 340 345 350	1056
65	GGC CGG ATC CGC AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser 355 360 365	1104
	AGC CTG CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG	1152

90

	Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu			
	370	375	380	
5	GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG CTG CGC Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg			1200
	385	390	395	400
10	CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG CGC CTG GAG ACC Leu Asp Ala Ile Thr Gly Pro Glu Glu Gly Arg Leu Glu Thr			1248
	405		410	415
15	ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC GTG GTG ATT CCC TCG GCG Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala			1296
	420		425	430
	ATC CCC ACC GAC CCG CGC AAC GTC GGC GGC GAC CTC GAC CCG TCC AGC Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser Ser			1344
	435		440	445
20	ATC CCC GAC AAG GAA CAG GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser			1392
	450		455	460
25	CAG CCC GGC AAA CCG CCG CGC GAG GACCTGAAG Gln Pro Gly Lys Pro Pro Arg Glu			1425
	465		470	
30	(2) INFORMATION FOR SEQ ID NO:8:			
	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 472 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
35	(ii) MOLECULE TYPE: protein			
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:			
40	Met Val Pro Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu			
	1	5	10	15

Lys Glu Lys Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn  
 20 25 30

5 Lys Thr Gln Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys  
 35 40 45

Ile Glu Val Lys Gly Glu Ala Val Lys Lys Glu Ala Ala Glu Lys  
 50 55 60

10 Leu Leu Glu Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile  
 65 70 75 80

Gly Gly Lys Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser  
 85 90 95

15 Leu Glu Ala Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly  
 100 105 110

20 Lys Asp Ala Leu Leu His Tyr Val Tyr Ala Lys Glu Gly Tyr  
 115 120 125

Glu Pro Val Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr  
 130 135 140

25 Glu Lys Ala Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg  
 145 150 155 160

Asp Ile Leu Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val  
 165 170 175

30 Leu Asn Thr Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu  
 180 185 190

Phe Thr Asn Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe  
 195 200 205

Leu Glu Gln Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe  
 210 215 220

40 Ala Tyr Tyr Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala  
 225 230 235 240

Pro Glu Ala Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn  
 245 250 255

45 Leu Thr Arg Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser  
 260 265 270

Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His  
 275 280 285

Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr  
 290 295 300

55 Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg  
 305 310 315 320

Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp  
 325 330 335

60 Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg  
 340 345 350

Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser  
 355 360 365

65 Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu  
 370 375 380

Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg

92

385	390	395	400
Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr			
	405	410	415
5	Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala		
	420	425	430
10	Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser Ser		
	435	440	445
Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser			
	450	455	460
15	Gln Pro Gly Lys Pro Pro Arg Glu		
	465	470	

## (2) INFORMATION FOR SEQ ID NO:9:

20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 1524 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

25           (ii) MOLECULE TYPE: DNA (genomic)

              (iii) HYPOTHETICAL: NO

30           (vi) ORIGINAL SOURCE:  
              (A) ORGANISM: *Bacillus anthracis*

              (ix) FEATURE:  
              (A) NAME/KEY: CDS  
              (B) LOCATION: 1..1524  
              (D) OTHER INFORMATION: /product=  
                          "LF(1-254) --TR--PE(362-613)"

35

40           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

	GCG GGC GGT CAT GGT GAT GTA GGT ATG CAC GTA AAA GAG AAA GAG AAA Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys 1 5 10 15	48
5	AAT AAA GAT GAG AAT AAG AGA AAA GAT GAA GAA CGA AAT AAA ACA CAG Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln 20 25 30	96
10	GAA GAG CAT TTA AAG GAA ATC ATG AAA CAC ATT GTA AAA ATA GAA GTA Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val 35 40 45	144
15	AAA GGG GAG GAA GCT GTT AAA AAA GAG GCA GCA GAA AAG CTA CTT GAG Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu 50 55 60	192
20	AAA GTA CCA TCT GAT GTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys 65 70 75 80	240
25	ATA TAT ATT GTG GAT GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala 85 90 95	288
30	TTA TCT GAA GAT AAG AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala 100 105 110	336
35	TTA TTA CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val 115 120 125	384
40	CTT GTA ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala 130 135 140	432
45	CTG AAC GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu 145 150 155 160	480
50	AGT AAA ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr 165 170 175	528
55	ATT AAA AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn 180 185 190	576
60	CAG CTT AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln 195 200 205	624
65	AAT AGC AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr 210 215 220	672
70	ATC GAG CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala 225 230 235 240	720
75	TTT AAT TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT CTA ACG CGT Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Thr Arg 245 250 255	768
80	GCG GCC AAC GCC GAC GTG GTG AGC CTG ACC TGC CCG GTC GCC GCC GGT Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala Gly 260 265 270	816
85	GAA TGC GCG GGC CCG GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC	864

	Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn			
	275	280	285	
5	TAT CCC ACT GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC			912
	Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe			
	290	295	300	
10	AGC ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG GCG			960
	Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala			
	305	310	315	320
15	CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC TAC CAC GGC			1008
	His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly			
	325	330	335	
20	ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC GGC GGG GTG CGC GCG			1056
	Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala			
	340	345	350	
25	CGC AGC CAG GAC CTC GAC GCG ATC TGG CGC GGT TTC TAT ATC GCC GGC			1104
	Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly			
	355	360	365	
30	GAT CCG GCG CTG GCC TAC GGC TAC GCC CAG GAC CAG GAA CCC GAC GCA			1152
	Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala			
	370	375	380	
35	CGC GGC CGG ATC CGC AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC			1200
	Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg			
	385	390	395	400
40	TCG AGC CTG CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG			1248
	Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro			
	405	410	415	
45	GAG GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG CTG			1296
	Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu			
	420	425	430	
50	CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG CGC CTG GAG			1344
	Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Gly Gly Arg Leu Glu			
	435	440	445	
55	ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC GTG GTG ATT CCC TCG			1392
	Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser			
	450	455	460	
60	GCG ATC CCC ACC GAC CCG CGC AAC GTC GGC GGC GAC CTC GAC CCG TCC			1440
	Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser			
	465	470	475	480
65	AGC ATC CCC GAC AAG GAA CAG GCG ATC AGC GCC CTG CCG GAC TAC GCC			1488
	Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala			
	485	490	495	
	AGC CAG CCC GGC AAA CCG CCG CGC GAG GAC CTG AAG			1524
	Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys			
	500	505		
	(2) INFORMATION FOR SEQ ID NO:10:			
	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 508 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
	(ii) MOLECULE TYPE: protein			

95

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gly Gly His Gly Asp Val Gly Met His Val Lys Glu Lys Glu Lys  
1 5 10 15

5 Asn Lys Asp Glu Asn Lys Arg Lys Asp Glu Glu Arg Asn Lys Thr Gln  
20 25 30

10 Glu Glu His Leu Lys Glu Ile Met Lys His Ile Val Lys Ile Glu Val  
35 40 45

Lys Gly Glu Glu Ala Val Lys Lys Glu Ala Ala Glu Lys Leu Leu Glu  
50 55 60

15 Lys Val Pro Ser Asp Val Leu Glu Met Tyr Lys Ala Ile Gly Gly Lys  
65 70 75 80

Ile Tyr Ile Val Asp Gly Asp Ile Thr Lys His Ile Ser Leu Glu Ala  
85 90 95

20 Leu Ser Glu Asp Lys Lys Ile Lys Asp Ile Tyr Gly Lys Asp Ala  
100 105 110

25 Leu Leu His Glu His Tyr Val Tyr Ala Lys Glu Gly Tyr Glu Pro Val  
115 120 125

Leu Val Ile Gln Ser Ser Glu Asp Tyr Val Glu Asn Thr Glu Lys Ala  
130 135 140

30

Leu Asn Val Tyr Tyr Glu Ile Gly Lys Ile Leu Ser Arg Asp Ile Leu  
 145 150 155 160

5 Ser Lys Ile Asn Gln Pro Tyr Gln Lys Phe Leu Asp Val Leu Asn Thr  
 165 170 175

Ile Lys Asn Ala Ser Asp Ser Asp Gly Gln Asp Leu Leu Phe Thr Asn  
 180 185 190

10 Gln Leu Lys Glu His Pro Thr Asp Phe Ser Val Glu Phe Leu Glu Gln  
 195 200 205

Asn Ser Asn Glu Val Gln Glu Val Phe Ala Lys Ala Phe Ala Tyr Tyr  
 210 215 220

15 Ile Glu Pro Gln His Arg Asp Val Leu Gln Leu Tyr Ala Pro Glu Ala  
 225 230 235 240

20 Phe Asn Tyr Met Asp Lys Phe Asn Glu Gln Glu Ile Asn Leu Thr Arg  
 245 250 255

Ala Ala Asn Ala Asp Val Val Ser Leu Thr Cys Pro Val Ala Ala Gly  
 260 265 270

25 Glu Cys Ala Gly Pro Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn  
 275 280 285

Tyr Pro Thr Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe  
 290 295 300

30 Ser Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala  
 305 310 315 320

His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr His Gly  
 325 330 335

Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe Gly Gly Val Arg Ala  
 340 345 350

40 Arg Ser Gln Asp Leu Asp Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly  
 355 360 365

Asp Pro Ala Leu Ala Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala  
 370 375 380

45 Arg Gly Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg  
 385 390 395 400

50 Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro  
 405 410 415

Glu Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro Leu  
 420 425 430

55 Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Arg Leu Glu  
 435 440 445

Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser  
 450 455 460

60 Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu Asp Pro Ser  
 465 470 475 480

Ser Ile Pro Asp Lys Glu Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala  
 485 490 495

65 Ser Gln Pro Gly Lys Pro Pro Arg Glu Asp Leu Lys  
 500 505

(2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2709 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

10

(iii) HYPOTHETICAL: NO

10

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

15

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2709
- (D) OTHER INFORMATION: /product= "PA(1-725) -----Human CD4 residues(1-178)"

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAA	GTT	AAA	CAG	GAG	AAC	CGG	TTA	TAA	AAT	GAA	TCA	GAA	TCA	AGT	TCC	48
Glu	Val	Lys	Gln	Glu	Asn	Arg	Leu	Leu	Asn	Glu	Ser	Glu	Ser	Ser	Ser	
1				5					10						15	
CAG	GGG	TTA	CTA	GGA	TAC	TAT	TTT	AGT	GAT	TTG	AAT	TTT	CAA	GCA	CCC	96
Gln	Gly	Leu	Leu	Gly	Tyr	Tyr	Phe	Ser	Asp	Leu	Asn	Phe	Gln	Ala	Pro	
					20				25				30			
ATG	GTG	GTT	ACC	TCT	TCT	ACT	ACA	GGG	GAT	TTA	TCT	ATT	CCT	AGT	TCT	144
Met	Val	Val	Thr	Ser	Ser	Thr	Thr	Gly	Asp	Leu	Ser	Ile	Pro	Ser	Ser	
					35				40			45				
GAG	TTA	GAA	AAT	ATT	CCA	TCG	GAA	AAC	CAA	TAT	TTT	CAA	TCT	GCT	ATT	192
Glu	Leu	Glu	Asn	Ile	Pro	Ser	Glu	Asn	Gln	Tyr	Phe	Gln	Ser	Ala	Ile	
					50				55			60				
TGG	TCA	GGA	TTT	ATC	AAA	GTT	AAG	AAG	AGT	GAT	GAA	TAT	ACA	TTT	GCT	240
Trp	Ser	Gly	Phe	Ile	Lys	Val	Lys	Lys	Ser	Asp	Glu	Tyr	Thr	Phe	Ala	
					65				70			75			80	
ACT	TCC	GCT	GAT	AAT	CAT	GTA	ACA	ATG	TGG	GTA	GAT	GAC	CAA	GAA	GTG	288
Thr	Ser	Ala	Asp	Asn	His	Val	Thr	Met	Trp	Val	Asp	Gln	Glu	Val		
					85				90			95				
ATT	AAT	AAA	GCT	TCT	AAT	TCT	AAC	AAA	ATC	AGA	TTA	GAA	AAA	GGA	AGA	336
Ile	Asn	Lys	Ala	Ser	Asn	Ser	Asn	Lys	Ile	Arg	Leu	Glu	Lys	Gly	Arg	
					100				105			110				
TTA	TAT	CAA	ATA	AAA	ATT	CAA	TAT	CAA	CGA	GAA	AAT	CCT	ACT	GAA	AAA	384
Leu	Tyr	Gln	Ile	Lys	Ile	Gln	Tyr	Gln	Arg	Glu	Asn	Pro	Thr	Glu	Lys	
					115				120			125				
GGA	TTG	GAT	TTC	AAG	TTG	TAC	TGG	ACC	GAT	TCT	CAA	AAT	AAA	AAA	GAA	432
Gly	Leu	Asp	Phe	Lys	Leu	Tyr	Trp	Thr	Asp	Ser	Gln	Asn	Lys	Lys	Glu	
					130				135			140				
GTG	ATT	TCT	AGT	GAT	AAC	TTA	CAA	TTG	CCA	GAA	TTA	AAA	CAA	AAA	TCT	480
Val	Ile	Ser	Ser	Asp	Asn	Leu	Gln	Leu	Pro	Glu	Leu	Lys	Gln	Lys	Ser	
					145				150			155			160	
TCG	AAC	TCA	AGA	AAA	AAG	CGA	AGT	ACA	AGT	GCT	GGA	CCT	ACG	GTT	CCA	528
Ser	Asn	Ser	Arg	Lys	Lys	Arg	Ser	Thr	Ser	Ala	Gly	Pro	Thr	Val	Pro	
					165				170			175				
GAC	CGT	GAC	AAT	GAT	GGA	ATC	CCT	GAT	TCA	TTA	GAG	GTA	GAA	GGA	TAT	576
Asp	Arg	Asp	Asn	Asp	Gly	Ile	Pro	Asp	Ser	Leu	Glu	Val	Glu	Gly	Tyr	
					180				185			190				

	ACG GTT GAT GTC AAA AAT AAA AGA ACT TTT CTT TCA CCA TGG ATT TCT Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser 195 200 205	624
5	AAT ATT CAT GAA AAG AAA GGA TTA ACC AAA TAT AAA TCA TCT CCT GAA Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu 210 215 220	672
10	AAA TGG AGC ACG GCT TCT GAT CCG TAC AGT GAT TTC GAA AAG GTT ACA Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr 225 230 235 240	720
15	GGA CGG ATT GAT AAG AAT GTA TCA CCA GAG GCA AGA CAC CCC CTT GTG Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val 245 250 255	768
20	GCA GCT TAT CCG ATT GTA CAT GTA GAT ATG GAG AAT ATT ATT CTC TCA Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser 260 265 270	816
25	AAA AAT GAG GAT CAA TCC ACA CAG AAT ACT GAT AGT GAA ACG AGA ACA Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr 275 280 285	864
30	ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA CAT ACT AGT GAA GTA CAT Ile Ser Lys Asn Thr Ser Arg Thr His Thr Ser Glu Val His 290 295 300	912
35	GGA AAT GCA GAA GTG CAT GCG TCG TTC TTT GAT ATT GGT GGG AGT GTA Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val 305 310 315 320	960
40	TCT GCA GGA TTT AGT AAT TCG AAT TCA AGT ACG GTC GCA ATT GAT CAT Ser Ala Gly Phe Ser Asn Ser Asn Ser Thr Val Ala Ile Asp His 325 330 335	1008
45	TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA ACA ATG GGT TTA Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu 340 345 350	1056
50	AAT ACC GCT GAT ACA GCA AGA TTA AAT GCC AAT ATT AGA TAT GTA AAT Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn 355 360 365	1104
55	ACT GGG ACG GCT CCA ATC TAC AAC GTG TTA CCA ACG ACT TCG TTA GTG Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val 370 375 380	1152
60	TTA GGA AAA AAT CAA ACA CTC GCG ACA ATT AAA GCT AAG GAA AAC CAA Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln 385 390 395 400	1200
65	TTA AGT CAA ATA CTT GCA CCT AAT AAT TAT TAT CCT TCT AAA AAC TTG Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu 405 410 415	1248
70	GCG CCA ATC GCA TTA AAT GCA CAA GAC GAT TTC AGT TCT ACT CCA ATT Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile 420 425 430	1296
75	ACA ATG AAT TAC AAT CAA TTT CTT GAG TTA GAA AAA ACG AAA CAA TTA Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu 435 440 445	1344
80	AGA TTA GAT ACG GAT CAA GTA TAT GGG AAT ATA GCA ACA TAC AAT TTT Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe 450 455 460	1392
85	GAA AAT GGA AGA GTG AGG GTG GAT ACA GGC TCG AAC TGG AGT GAA GTG	1440

99

	Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val		
	465 470 475 480		
5	TTA CCG CAA ATT CAA GAA ACA ACT GCA CGT ATC ATT TTT AAT GGA AAA Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys		1488
	485 490 495		
10	GAT TTA AAT CTG GTA GAA AGG CGG ATA GCG GCG GTT AAT CCT AGT GAT Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp		1536
	500 505 510		
15	CCA TTA GAA ACG ACT AAA CCG GAT ATG ACA TTA AAA GAA GCC CTT AAA Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys		1584
	515 520 525		
20	ATA GCA TTT GGA TTT AAC GAA CCG AAT GGA AAC TTA CAA TAT CAA GGG Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly		1632
	530 535 540		
25	AAA GAC ATA ACC GAA TTT GAT TTT AAT TTC GAT CAA CAA ACA TCT CAA Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln		1680
	545 550 555 560		
30	AAT ATC AAG AAT CAG TTA GCG GAA TTA AAC GCA ACT AAC ATA TAT ACT Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr		1728
	565 570 575		
35	GTA TTA GAT AAA ATC AAA TTA AAT GCA AAA ATG AAT ATT TTA ATA AGA Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg		1776
	580 585 590		
40	GAT AAA CGT TTT CAT TAT GAT AGA AAT AAC ATA GCA GTT GGG GCG GAT Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp		1824
	595 600 605		
45	GAG TCA GTA GTT AAG GAG GCT CAT AGA GAA GTA ATT AAT TCG TCA ACA Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr		1872
	610 615 620		
50	GAG GGA TTA TTG TTA AAT ATT GAT AAG GAT ATA AGA AAA ATA TTA TCA Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser		1920
	625 630 635 640		
55	GGT TAT ATT GTA GAA ATT GAA GAT ACT GAA GGG CTT AAA GAA GTT ATA Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile		1968
	645 650 655		
60	AAT GAC AGA TAT GAT ATG TTG AAT ATT TCT AGT TTA CGG CAA GAT GGA Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly		2016
	660 665 670		
65	AAA ACA TTT ATA GAT TTT AAA AAA TAT AAT GAT AAA TTA CCG TTA TAT Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr		2064
	675 680 685		
70	ATA AGT AAT CCC AAT TAT AAG GTA AAT GTA TAT GCT GTT ACT AAA GAA Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu		2112
	690 695 700		
75	AAC ACT ATT ATT AAT CCT AGT GAG AAT GGG GAT ACT AGT ACC AAC GGG Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly		2160
	705 710 715 720		
80	ATC AAG AAA ATT TTA AAG AAA GTG GTG CTG GGC AAA AAA GGG GAT ACA Ile Lys Lys Ile Leu Lys Val Val Leu Gly Lys Lys Gly Asp Thr		2208
	725 730 735		
85	GTG GAA CTG ACC TGT ACA GCT TCC CAG AAG AAG AGC ATA CAA TTC CAC Val Glu Leu Thr Cys Thr Ala Ser Gln Lys Lys Ser Ile Gln Phe His		2256

	740	745	750	
5	TGG AAA AAC TCC AAC CAG ATA AAG ATT CTG GGA AAT CAG GGC TCC TTC Trp Lys Asn Ser Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser Phe 755 760 765			2304
10	TTA ACT AAA GGT CCA TCC AAG CTG AAT GAT CGC GCT GAC TCA AGA AGA Leu Thr Lys Gly Pro Ser Lys Leu Asn Asp Arg Ala Asp Ser Arg Arg 770 775 780			2352
15	AGC CTT TGG GAC CAA GGA AAC TTC CCC CTG ATC ATC AAG AAT CTT AAG Ser Leu Trp Asp Gln Gly Asn Phe Pro Leu Ile Ile Lys Asn Leu Lys 785 790 795 800			2400
20	ATA GAA GAC TCA GAT ACT TAC ATC TGT GAA GTG GAG GAC CAG AAG GAG Ile Glu Asp Ser Asp Thr Tyr Ile Cys Glu Val Glu Asp Gln Lys Glu 805 810 815			2448
25	GAG GTG CAA TTG CTA GTG TTC GGA TTG ACT GCC AAC TCT GAC ACC CAC Glu Val Gln Leu Leu Val Phe Gly Leu Thr Ala Asn Ser Asp Thr His 820 825 830			2496
30	CTG CTT CAG GGG CAG AGC CTG ACC CTG ACC TTG GAG AGC CCC CCT GGT Leu Leu Gln Gly Gln Ser Leu Thr Leu Thr Leu Glu Ser Pro Pro Gly 835 840 845			2544
35	AGT AGC CCC TCA GTG CAA TGT AGG AGT CCA AGG GGT AAA AAC ATA CAG Ser Ser Pro Ser Val Gln Cys Arg Ser Pro Arg Gly Lys Asn Ile Gln 850 855 860			2592
40	GGG GGG AAG ACC CTC TCC GTG TCT CAG CTG GAG CTC CAG GAT AGT GGC Gly Gly Lys Thr Leu Ser Val Ser Gln Leu Glu Leu Gln Asp Ser Gly 865 870 875 880			2640
	ACC TGG ACA TGC ACT GTC TTG CAG AAC CAG AAG AAG GTG GAG TTC AAA Thr Trp Thr Cys Thr Val Leu Gln Asn Gln Lys Lys Val Glu Phe Lys 885 890 895			2688
	ATA GAC ATC GTG GTG CTA GCT Ile Asp Ile Val Val Leu Ala 900			2709

(2) INFORMATION FOR SEQ ID NO:12:

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

55 Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser  
1 5 10 15

101

Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro  
 20 25 30  
 Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser  
 5 35 40 45  
 Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile  
 50 55 60  
 Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala  
 10 65 70 75 80  
 Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val  
 85 90 95  
 15 Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg  
 100 105 110  
 Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys  
 20 115 120 125  
 Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu  
 130 135 140  
 25 Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser  
 145 150 155 160  
 Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro  
 165 170 175  
 30 Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr  
 180 185 190  
 Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser  
 35 195 200 205  
 Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu  
 210 215 220  
 40 Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr  
 225 230 235 240  
 Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val  
 245 250 255  
 45 Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser  
 260 265 270  
 Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr  
 50 275 280 285  
 Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His  
 290 295 300  
 55 Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val  
 305 310 315 320  
 Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His  
 325 330 335  
 60 Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu  
 340 345 350  
 Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn  
 65 355 360 365  
 Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val  
 370 375 380  
 Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln

102

	385	390	395	400
	Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu			
	405		410	415
5	Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile			
	420		425	430
10	Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu			
	435		440	445
	Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe			
	450		455	460
15	Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val			
	465		470	475
	Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys			
	485		490	495
20	Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp			
	500		505	510
25	Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys			
	515		520	525
	Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly			
	530		535	540
30	Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln			
	545		550	555
	Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr			
	565		570	575
35	Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg			
	580		585	590
40	Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp			
	595		600	605
	Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr			
	610		615	620
45	Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser			
	625		630	635
	Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile			
	645		650	655
50	Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly			
	660		665	670
	Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr			
	675		680	685
55	Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu			
	690		695	700
60	Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly			
	705		710	720
	Ile Lys Lys Ile Leu Lys Lys Val Val Leu Gly Lys Lys Gly Asp Thr			
	725		730	735
65	Val Glu Leu Thr Cys Thr Ala Ser Gln Lys Lys Ser Ile Gln Phe His			
	740		745	750
	Trp Lys Asn Ser Asn Gln Ile Lys Ile Leu Gly Asn Gln Gly Ser Phe			
	755		760	765

103

Leu Thr Lys Gly Pro Ser Lys Leu Asn Asp Arg Ala Asp Ser Arg Arg  
770 775 780

5 Ser Leu Trp Asp Gln Gly Asn Phe Pro Leu Ile Ile Lys Asn Leu Lys  
785 790 795 800

Ile Glu Asp Ser Asp Thr Tyr Ile Cys Glu Val Glu Asp Gln Lys Glu  
805 810 815

10 Glu Val Gln Leu Leu Val Phe Gly Leu Thr Ala Asn Ser Asp Thr His  
820 825 830

Leu Leu Gln Gly Gln Ser Leu Thr Leu Thr Leu Glu Ser Pro Pro Gly  
835 840 845

15 Ser Ser Pro Ser Val Gln Cys Arg Ser Pro Arg Gly Lys Asn Ile Gln  
850 855 860

Gly Gly Lys Thr Leu Ser Val Ser Gln Leu Glu Leu Gln Asp Ser Gly  
865 870 875 880

20 Thr Trp Thr Cys Thr Val Leu Gln Asn Gln Lys Lys Val Glu Phe Lys  
885 890 895

25 Ile Asp Ile Val Val Leu Ala  
900

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

15

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..8
- (D) OTHER INFORMATION: /label= PAHIV

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

25

Ser	Gln	Asn	Tyr	Pro	Val	Val	Gln
1				5			

## (2) INFORMATION FOR SEQ ID NO:14:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

40

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus anthracis

45

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..12
- (D) OTHER INFORMATION: /label= PAHIV-1

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

55

Gln	Val	Ser	Gln	Asn	Tyr	Pro	Ile	Val	Gln	Asn	Ile
1				5					10		

## (2) INFORMATION FOR SEQ ID NO:15:

60

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

65

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Bacillus anthracis*

5 (ix) FEATURE:  
(A) NAME/KEY: Peptide  
(B) LOCATION: 1..12  
(D) OTHER INFORMATION: /label= PAHIV-2

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe  
1 5 10

15 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
20 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25 (iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

30 (vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Bacillus anthracis*

(ix) FEATURE:  
(A) NAME/KEY: Peptide  
(B) LOCATION: 1..12  
35 (D) OTHER INFORMATION: /label= PAHIV-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

40 Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp  
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

55 (vi) ORIGINAL SOURCE:  
(A) ORGANISM: *Bacillus anthracis*

60 (ix) FEATURE:  
(A) NAME/KEY: Peptide  
(B) LOCATION: 1..13  
(D) OTHER INFORMATION: /label= PAHIV-4

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:18:

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 45 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Bacillus anthracis*

20 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 3..44  
 (D) OTHER INFORMATION: /product= "Primer 1A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

25 CG CAA GTA TCA CAA AAT TAT CCG ATC GTG CAA AAC ATA CTG CAG  
 Gln Val Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Leu Gln  
 1 5 10

44

G 45  
 (2) INFORMATION FOR SEQ ID NO:19:

30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 14 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40 Gln Val Ser Gln Asn Tyr Pro Ile Val Gln Asn Ile Leu Gln  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:20:

45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 46 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

55 (iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Bacillus anthracis*

60 (ix) FEATURE:  
 (A) NAME/KEY: misc\_feature  
 (B) LOCATION: 1..46  
 (D) OTHER INFORMATION: /product= "PRIMER 1B"

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTTCTGCAG TATGTTTGC ACGATCGGAT AATTTGTGA TACTTG

46

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

10 (iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

## 15 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..44
- (D) OTHER INFORMATION: /product= "Primer 2A"

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

25 CG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG  
Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln  
1 5 10

44

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## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln  
1 5 10

45

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

55

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

## 60 (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..46
- (D) OTHER INFORMATION: /product= "PRIMER 2B"

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

108

GTCCCTGCAG AAAATTACCA CGTTGCATCA TGATAGTGGC AGTGGT

46

## (2) INFORMATION FOR SEQ ID NO:24:

5       (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 45 base pairs  
          (B) TYPE: nucleic acid  
          (C) STRANDEDNESS: single  
          (D) TOPOLOGY: linear

10      (ii) MOLECULE TYPE: DNA (genomic)

15      (iii) HYPOTHETICAL: NO

15      (vi) ORIGINAL SOURCE:  
          (A) ORGANISM: *Bacillus anthracis*

20      (ix) FEATURE:  
          (A) NAME/KEY: CDS  
          (B) LOCATION: 3..44  
          (D) OTHER INFORMATION: /product= "Primer 3A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

25      CG ACT GTC TCT TTT AAC TTC CCG CAA ATC ACG CTT TGG CTG CAG  
          Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Leu Gln  
          1                   5                   10

44

30      G    45

## (2) INFORMATION FOR SEQ ID NO:25:

35      (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 14 amino acids  
          (B) TYPE: amino acid  
          (D) TOPOLOGY: linear

40      (ii) MOLECULE TYPE: protein

45      (xii) SEQUENCE DESCRIPTION: SEQ ID NO:25:  
          Thr Val Ser Phe Asn Phe Pro Gln Ile Thr Leu Trp Leu Gln  
          1                   5                   10

## (2) INFORMATION FOR SEQ ID NO:26:

50      (i) SEQUENCE CHARACTERISTICS:  
          (A) LENGTH: 46 base pairs  
          (B) TYPE: nucleic acid  
          (C) STRANDEDNESS: single  
          (D) TOPOLOGY: linear

55      (ii) MOLECULE TYPE: DNA (genomic)

60      (iii) HYPOTHETICAL: NO

60      (iv) ANTI-SENSE: YES

65      (vi) ORIGINAL SOURCE:  
          (A) ORGANISM: *Bacillus anthracis*

65      (ix) FEATURE:  
          (A) NAME/KEY: misc\_feature  
          (B) LOCATION: 1..46  
          (D) OTHER INFORMATION: /product= "PRIMER 3B"

109

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCCTGCAG CCAAAGCGTG ATTTGCAGGA AGTTAAAAGA GACAGT

46

## 5 (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

## 20 (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3..47
- (D) OTHER INFORMATION: /product= "Primer 4A"

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CG GGC GGT TCT GCC TTT AAC TTC CCG ATC GTC ATG GGA GGT CTG CAG  
Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly Leu Gln  
1 5 10 15

47

30 G

48

## 35 (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

110

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Ser Ala Phe Asn Phe Pro Ile Val Met Gly Gly Leu Gln  
1 5 10 15

5

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

20 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

## (ix) FEATURE:

- (A) NAME/KEY: *misc\_feature*
- (B) LOCATION: 1..49
- (D) OTHER INFORMATION: /product= "PRIMER 4B"

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTCCCTGCAG ACCTCCCATG ACGATCGGGA AGTTAAAGGC AGAACCGCC

49

## (2) INFORMATION FOR SEQ ID NO:30:

## 35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2160 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

45 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Bacillus anthracis*

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2157
- (D) OTHER INFORMATION: /product= "PAHIV#2"

111

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

	GAA GTT AAA CAG GAG AAC CGG TTA TTA AAT GAA TCA GAA TCA AGT TCC	48
5	Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser	
	1 5 10 15	
	CAG GGG TTA CTA GGA TAC TAT TTT AGT GAT TTG AAT TTT CAA GCA CCC	96
	Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro	
	20 25 30	
10	ATG GTG GTT ACC TCT TCT ACT ACA GGG GAT TTA TCT ATT CCT AGT TCT	144
	Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser	
	35 40 45	
15	GAG TTA GAA AAT ATT CCA TCG GAA AAC CAA TAT TTT CAA TCT GCT ATT	192
	Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile	
	50 55 60	
20	TGG TCA GGA TTT ATC AAA GTT AAG AAG AGT GAT GAA TAT ACA TTT GCT	240
	Trp Ser Gly Phe Ile Lys Val Lys Ser Asp Glu Tyr Thr Phe Ala	
	65 70 75 80	
25	ACT TCC GCT GAT AAT CAT GTA ACA ATG TGG GTA GAT GAC CAA GAA GTG	288
	Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val	
	85 90 95	
30	ATT AAT AAA GCT TCT AAT TCT AAC AAA ATC AGA TTA GAA AAA GGA AGA	336
	Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg	
	100 105 110	
	TTA TAT CAA ATA AAA ATT CAA TAT CAA CGA GAA AAT CCT ACT GAA AAA	384
	Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys	
	115 120 125	
35	GGA TTG GAT TTC AAG TTG TAC TGG ACC GAT TCT CAA AAT AAA AAA GAA	432
	Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu	
	130 135 140	
40	GTG ATT TCT AGT GAT AAC TTA CAA TTG CCA GAA TTA AAA CAA AAA TCT	480
	Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser	
	145 150 155 160	
45	TCG AAC ACT GCC ACT ATC ATG ATG CAA CGT GGT AAT TTT CTG CAG GGA	528
	Ser Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln Gly	
	165 170 175	
	CCT ACG GTT CCA GAC CGT GAC AAT GAT GGA ATC CCT GAT TCA TTA GAG	576
	Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu	
	180 185 190	
50	GTA GAA GGA TAT ACG GTT GAT GTC AAA AAT AAA AGA ACT TTT CTT TCA	624
	Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser	
	195 200 205	

112

	CCA TGG ATT TCT AAT ATT CAT GAA AAG AAA GGA TTA ACC AAA TAT AAA Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys 210 215 220	672
5	TCA TCT CCT GAA AAA TGG AGC ACG GCT TCT GAT CCG TAC AGT GAT TTC Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe 225 230 235 240	720
10	GAA AAG GTT ACA GGA CGG ATT GAT AAG AAT GTA TCA CCA GAG GCA AGA Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg 245 250 255	768
15	CAC CCC CTT GTG GCA GCT TAT CCG ATT GTA CAT GTA GAT ATG GAG AAT His Pro Leu Val Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn 260 265 270	816
20	ATT ATT CTC TCA AAA AAT GAG GAT CAA TCC ACA CAG AAT ACT GAT AGT Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser 275 280 285	864
25	GAA ACG AGA ACA ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA CAT ACT Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr 290 295 300	912
30	AGT GAA GTA CAT GGA AAT GCA GAA GTG CAT GCG TCG TTC TTT GAT ATT Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile 305 310 315 320	960
35	GGT GGG AGT GTA TCT GCA GGA TTT AGT AAT TCG AAT TCA AGT ACG GTC Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val 325 330 335	1008
40	GCA ATT GAT CAT TCA CTA TCT CTA GCA GGG GAA AGA ACT TGG GCT GAA Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu 340 345 350	1056
45	ACA ATG GGT TTA AAT ACC GCT GAT ACA GCA AGA TTA AAT GCC AAT ATT Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile 355 360 365	1104
50	AGA TAT GTA AAT ACT GGG ACG GCT CCA ATC TAC AAC GTG TTA CCA ACG Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr 370 375 380	1152
55	ACT TCG TTA GTG TTA GGA AAA AAT CAA ACA CTC GCG ACA ATT AAA GCT Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala 385 390 395 400	1200
60	AAG GAA AAC CAA TTA AGT CAA ATA CTT GCA CCT AAT AAT TAT TAT CCT Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro 405 410 415	1248
65	TCT AAA AAC TTG GCG CCA ATC GCA TTA AAT GCA CAA GAC GAT TTC AGT Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser 420 425 430	1296
70	TCT ACT CCA ATT ACA ATG AAT TAC GGG AAT ATA GCA ACA TAC AAT TTT Ser Thr Pro Ile Thr Met Asn Tyr Gly Asn Ile Ala Thr Tyr Asn Phe 435 440 445	1344
75	GAA AAT GGA AGA GTG AGG GTG GAT ACA GGC TCG AAC TGG AGT GAA GTG Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val 450 455 460	1392
80	TTA CCG CAA ATT CAA GAA ACA ACT GCA CGT ATC ATT TTT AAT GGA AAA Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys 465 470 475 480	1440
85	GAT TTA AAT CTG GTA GAA AGG CGG ATA GCG GCG GTT AAT CCT AGT GAT	1488

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	Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp	485	490	495	
5	CCA TTA GAA ACG ACT AAA CCG GAT ATG ACA TTA AAA GAA GCC CTT AAA Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys	500	505	510	1536
10	ATA GCA TTT GGA TTT AAC GAA CCG AAT GGA AAC TTA CAA TAT CAA GGG Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly	515	520	525	1584
15	AAA GAC ATA ACC GAA TTT GAT TTT AAT TTC GAT CAA CAA ACA TCT CAA Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln	530	535	540	1632
20	AAT ATC AAG AAT CAG TTA GCG GAA TTA AAC GCA ACT AAC ATA TAT ACT Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr	545	550	555	1680
25	GTA TTA GAT AAA ATC AAA TTA AAT GCA AAA ATG AAT ATT TTA ATA AGA Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg	565	570	575	1728
30	GAT AAA CGT TTT CAT TAT GAT AGA AAT AAC ATA GCA GTT GGG GCG GAT Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp	580	585	590	1776
35	GAG TCA GTA GTT AAG GAG GCT CAT AGA GAA GTA ATT AAT TCG TCA ACA Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr	595	600	605	1824
40	GAG GGA TTA TTG TTA AAT ATT GAT AAG GAT ATA AGA AAA ATA TTA TCA Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser	610	615	620	1872
45	GGT TAT ATT GTA GAA ATT GAA GAT ACT GAA GGG CTT AAA GAA GTT ATA Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile	625	630	635	1920
50	AAT GAC AGA TAT GAT ATG TTG AAT ATT TCT AGT TTA CGG CAA GAT GGA Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly	645	650	655	1968
55	AAA ACA TTT ATA GAT TTT AAA AAA TAT AAT GAT AAA TTA CCG TTA TAT Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr	660	665	670	2016
60	ATA AGT AAT CCC AAT TAT AAG GTA AAT GTA TAT GCT GTT ACT AAA GAA Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu	675	680	685	2064
65	AAC ACT ATT ATT AAT CCT AGT GAG AAT GGG GAT ACT AGT ACC AAC GGG Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly	690	695	700	2112
	ATC AAG AAA ATT TTA ATC TTT TCT AAA AAA GGC TAT GAG ATA GGA Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly	705	710	715	2157
	TAA				2160

## (2) INFORMATION FOR SEQ ID NO:31:

65 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 719 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

114

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

5	Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser			
	1	5	10	15
	Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro			
	20	25	30	
10	Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser			
	35	40	45	
15	Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile			
	50	55	60	
	Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala			
	65	70	75	80
20	Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val			
	85	90	95	
	Ile Asn Lys Ala Ser Asn Ser Asn Ile Arg Leu Glu Lys Gly Arg			
	100	105	110	
25	Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys			
	115	120	125	
30	Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu			
	130	135	140	
	Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser			
	145	150	155	160
35	Ser Asn Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Leu Gln Gly			
	165	170	175	
	Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu			
	180	185	190	
40	Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser			
	195	200	205	
	Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys			
	210	215	220	
45	Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe			
	225	230	235	240
50	Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg			
	245	250	255	
	His Pro Leu Val Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn			
	260	265	270	
55	Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser			
	275	280	285	
	Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr			
	290	295	300	
60	Ser Glu Val His Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile			
	305	310	315	320
65	Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val			
	325	330	335	
	Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu			
	340	345	350	

Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile  
 355 360 365  
 5 Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr  
 370 375 380  
 Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala  
 385 390 395 400  
 10 Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro  
 405 410 415  
 Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser  
 420 425 430  
 15 Ser Thr Pro Ile Thr Met Asn Tyr Gly Asn Ile Ala Thr Tyr Asn Phe  
 435 440 445  
 Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val  
 450 455 460  
 20 Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys  
 465 470 475 480  
 25 Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp  
 485 490 495  
 Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys  
 500 505 510  
 30 Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly  
 515 520 525  
 Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln  
 530 535 540  
 35 Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr  
 545 550 555 560  
 40 Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg  
 565 570 575  
 Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp  
 580 585 590  
 45 Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr  
 595 600 605  
 Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser  
 610 615 620  
 50 Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile  
 625 630 635 640  
 Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly  
 645 650 655  
 Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr  
 660 665 670  
 60 Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu  
 675 680 685  
 Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly  
 690 695 700  
 65 Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly  
 705 710 715

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a fusion protein, comprising a nucleotide sequence encoding the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein and a nucleotide sequence encoding an activity inducing domain of a second protein.

2. The nucleic acid of claim 1, wherein the second protein is a toxin.

3. The nucleic acid of claim 2, wherein the toxin is *Pseudomonas exotoxin A*.

4. The nucleic acid of claim 2, wherein the toxin is the A chain of *Diphtheria* toxin.

5. The nucleic acid of claim 2, wherein the toxin is shiga toxin.

6. The nucleic acid of claim 1, comprising the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:5.

7. The nucleic acid of claim 1, comprising the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:6.

8. A protein encoded by the nucleic acid of claim 1.

9. A vector comprising the nucleic acid of claim 1.

10. The vector of claim 9 in a host capable of expressing the protein encoded by the nucleic acid.

11. A nucleic acid encoding a fusion protein, the nucleic acid comprising a nucleotide sequence encoding the translocation domain and anthrax lethal factor (LF) binding

domain of native anthrax protective antigen (PA) protein and a nucleotide sequence encoding a ligand domain which specifically binds a cellular target.

5           12. The nucleic acid of claim 11, wherein the ligand domain specifically binds to an HIV protein expressed on the surface of an HIV-infected cell.

10          13. The nucleic acid of claim 11, wherein the ligand domain is a growth factor.

15          14. The nucleic acid of claim 11, wherein the nucleotide sequence encoding the translocation domain and LF binding domain of the native PA protein further comprises the nucleotide sequence encoding the remainder of the native PA protein.

20          15. A protein encoded by the nucleic acid of claim 11.

25          16. A vector comprising the nucleic acid of claim 11.

30          17. The vector of claim 16 in a host capable of expressing the protein encoded by the nucleic acid.

25          18. A method of killing a tumor cell in a subject, the method comprising the steps of:

30          a) administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a tumor cell specific ligand domain in an amount sufficient to bind to a tumor cell; and

35          b) administering to the subject a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the tumor cell and kills the tumor cell.

19. A method of killing HIV-infected cells in a subject, the method comprising the steps of:

a) administering to the subject a first fusion protein comprising the translocation domain and LF binding domain of the native PA protein and a ligand domain that specifically binds to an HIV protein expressed on the surface of an HIV-infected cell in an amount sufficient to bind to an HIV-infected cell; and

b) administering to the subject a second fusion protein comprising the PA binding domain of the native LF protein and a cytotoxic domain of a non-LF protein in an amount sufficient to bind to the first protein, whereby the second protein is internalized into the HIV-infected cell and kills the HIV-infected cell, thereby preventing propagation of HIV.

20. A method for delivering an activity to a cell comprising the steps of:

a) administering to the cell a protein comprising the translocation domain and the LF binding domain of the native PA protein and a ligand domain; and

b) administering to the cell a compound comprising the PA binding domain of the native LF protein chemically attached to an activity inducing moiety, whereby the compound administered in step b) is internalized into the cell and effects the activity within the cell.

21. The method of claim 20, wherein the ligand domain is the receptor binding domain of the native PA protein.

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22. The method of claim 20, wherein the activity inducing moiety is a polypeptide.

35 23. The method of claim 22, wherein the polypeptide is a growth factor.

24. The method of claim 20, wherein the activity inducing moiety is an antisense nucleic acid.

25. The method of claim 20, wherein the activity inducing moiety is a nucleic acid encoding a desired gene product.

5        26. A compound comprising the PA binding domain of the native LF protein chemically attached to a non-LF activity inducing moiety.

10      27. The composition of claim 26, wherein the activity inducing moiety is a polypeptide.

28. The composition of claim 26, wherein the activity inducing moiety is a radioisotope.

15      29. The composition of claim 26, wherein the activity inducing moiety is an antisense nucleic acid.

20      30. The composition of claim 26, wherein the activity inducing moiety is a nucleic acid encoding a desired gene product.

31. The nucleic acid of claim 11, comprising the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:11.

25      32. A nucleic acid comprising a nucleotide sequence encoding an anthrax protective antigen which is altered to include a cleavage site recognized by a protease produced by an intracellular pathogen.

30      33. The nucleic acid of claim 32 wherein the intracellular pathogen is a virus.

35      34. The nucleic acid of claim 33 wherein the alteration comprises a mutation in at least one of amino acid residues 164-167 (the trypsin cleavage site).

120

35. The nucleic acid of claim 34 wherein the virus is a retrovirus.

36. The nucleic acid of claim 35 wherein the  
5 retrovirus is an HIV.

37. The nucleic acid of claim 36 wherein the amino acids at residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, QVSQNYPIVQNI, TVSFNFPQITLW, and GGSAFNFPIVMGG.  
10

38. A polypeptide comprising an amino acid sequence encoding an anthrax protective antigen which is altered to include a cleavage site recognized by a protease produced by a  
15 retrovirus.

39. The polypeptide of claim 38 wherein the alteration comprises a mutation in at least one of amino acid residues 164-167 (the trypsin cleavage site).  
20

40. The polypeptide of claim 39 wherein the retrovirus is an HIV.

41. The polypeptide of claim 40 wherein the amino acid residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, QVSQNYPIVQNI, TVSFNFPQITLW, and GGSAFNFPIVMGG.  
25

42. A method of killing a cell which is infected with an intracellular pathogen, the method comprising:  
30

applying to the cell a composition comprising an effective amount an altered anthrax protective antigen (PA) having a cleavage site recognized by a protease produced by the intracellular pathogen.  
35

43. The method of claim 42 wherein the cleavage site is at amino acid residues 164-167.

44. The method of claim 42 wherein the intracellular pathogen is a virus.

5 45. The method of claim 44 wherein the virus is a retrovirus.

46. A method of claim 45 wherein the retrovirus is an HIV.

10 47. The method of claim 46 wherein the amino acids at residues 164-167 are replaced with an amino acid sequence selected from the group comprising NTATIMMQRGNF, QVSQNYPIVQNI, TVSFNFPQITLW, and GGSAFNFPQIVMGG.

15 48. The method of claim 42 wherein the cell is harbored in a human.

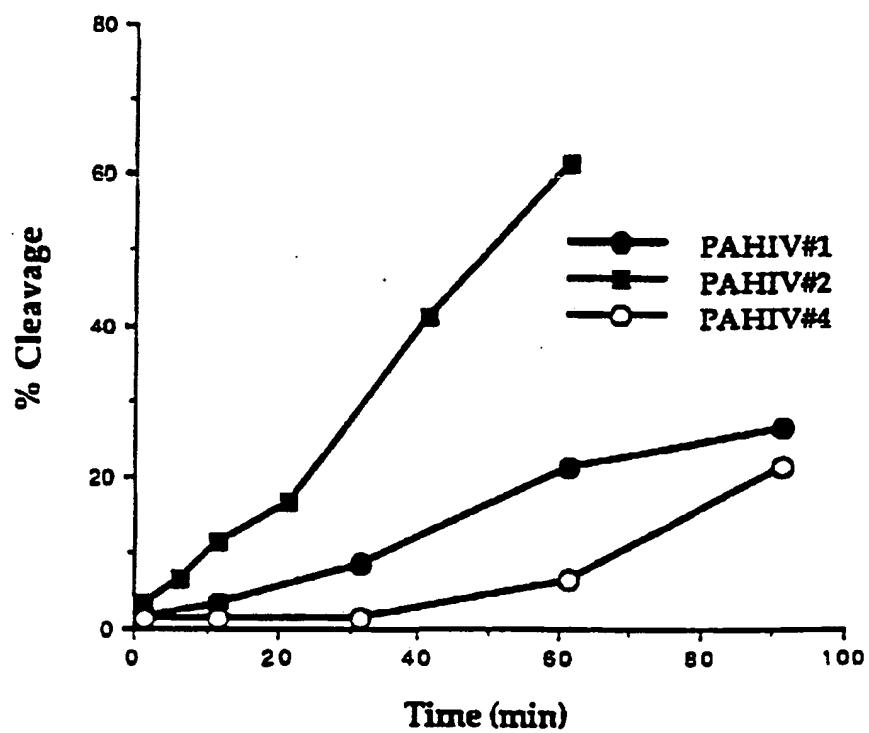
20 49. The method of claim 48 wherein the step of applying the composition includes parenterally administering the composition to the human.

50. The method of claim 49 wherein the parenteral administration is intravenous.

25 51. The method of claim 48 wherein the effective amount of altered protective antigen is from about 5 to about 25 micrograms per kilogram of body weight of a human harboring the infected cell.

30 52. The method of claim 51 wherein the effective amount of altered protective antigen is about 10 micrograms per kilogram of body weight of a human harboring the infected cell.

Figure 1

**Cleavage of mutant PAHIV proteins with purified HIV-1 protease**

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